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ANALYSIS OF THE KU ANTIGEN-DEPENDENT ACTIVATION OF REPORTER GENES IN YEAST AND CHARACTERIZATION OF THE NUCLEAR IMPORT SIGNALS OF KU ANTIGEN

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ABSTRACT

Ku antigen (Ku70/Ku80) is a heteromeric, DNA binding protein that has been implicated in DNA repair, V(D)J recombination, tumour suppression, growth, cell cycle control, telomere maintenance and the regulation of gene transcription. Ku is a DNA binding subunit of the DNA-dependent protein kinase and functions to recruit and stimulate the catalytic subunit at appropriate DNA target sites. I present the unexpected finding that expression of human Ku fused to an ectopic activation domain broadly activated reporter genes integrated into the yeast genome independent of the reporter gene, yeast minimal promoter and reporter gene integration site. Interestingly, expression of various Ku70 deletion mutants alone or in combination with Ku80 revealed that the regions of Ku identified to be required for activation share a striking resemblance to portions of Ku needed to bind DNA. Furthermore, a mutation in Ku70 that negates the Ku70-dependent activation property in yeast also disrupts the ability of Ku70 monomers to associate tightly with the nucleus in mammalian cells. Moreover, I demonstrate that human Ku80 cannot substitute for the yeast homologue (HDF2) to rescue the temperature sensitive phenotype of a yeast strain deficient in Ku80 but expression of a variant human Ku heterodimer partially rescues the phenotype. By contrast, the characteristic slow growth displayed by HDF2 deficient yeast at 30 °C could not be rescued by expression of the same human Ku heterodimer. Also, I determined that Ku70 contains a bipartite nuclear localization signal between amino acids 539-556, while the core of the nuclear localization signal of Ku80 is a basic motif from 565-

569. Each nuclear localization signal mediated the nuclear transfer of the individual Ku subunits and Ku heterodimer, while site directed mutagenesis of both nuclear localization signals resulted in a Ku heterodimer that was localized to the cytoplasm. Lastly, I show that mutations in Ku previously proposed to abrogate Ku70/Ku80 heterodimerization reduce the accumulation of Ku70 without affecting heterodimer formation in mammalian cells.

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ABBREVIATIONS

Å angstrom

AD activation domain

ADKu70 activation domain Ku70

ADKu80 activation domain Ku80

AMT1 aminomethyltransferase 1

3-AT 3-amino-1,2,4-triazole

ATM ataxia-telangiectasia-mutated

ATP adenosine triphosphate

ATR ataxia-telangiectasia and Rad3 related protein

bps base pairs

BSA bovine serum albumin

BUR base unpairing region

C cytoplasmic

CAS cellular apoptosis susceptibility

CD cluster of differentiation

cDNA complementary DNA

COL3A1 collagene 3A1

Crm1 chromosome region maintenance protein 1

C-terminus carboxy terminus

CsCl cesium chloride

CYC1 cytochrome c-1

DBD DNA binding domain

DI dimerization interface

Dlx2 distal-less homeo box 2

DMEM Dulbecco's modified Eagle medium

DMSO dimethyl sulphoxide

DNA deoxyribonucleic acid

DNaseI deoxyribonuclease I

DNA-PK DNA-dependent protein kinase

DNA-PKcs DNA-dependent protein kinase catalytic subunit

DSB double-stranded break

DTT dithiothreitol

ECL enhanced chemiluminescence

E. coli *Escherichia coli*

EDTA ethylenediamine tetraacetic acid

e.g. example given

EMSA electrophoretic mobility shift assay

EGFP enhanced green fluorescent protein

EtBr ethidium bromide

FBS fetal bovine serum

FITC fluorescein isothiocyanate

FRAP fluorescence recovery after photobleaching

g gravity

G_1 gap_1

GAL1 galactose 1

Gal4AD Gal4 activation domain

Gal4DBD Gal4 DNA binding domain

GCN5 general control of amino acid synthesis protein 5

GDP guanosine diphosphate

GKu70 EGFPKu70

GKu80 EGFPKu80

G_2/M gap_2/mitosis

GPB glycophorin B

GPD glyceraldehyde-3-phosphate dehydrogenase

GR glucocorticoid receptor

GST glutathione S-transferase

GTE glucose/tris/EDTA

GTP guanosine triphosphate

h hours

hKu70 human Ku70

hKu80 human Ku80

HA hemagglutinin

HAT histone acetyl transferase

HDF1 high affinity DNA binding factor 1

HDF2 high affinity DNA binding factor 2

HEJ homologous end joining

HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HIS3 histidine 3

HIV-1 human immunodeficiency virus 1

hnRNP K heterogeneous nuclear ribonucleoprotein K

HOXC4 homeo box C4

HR homologous recombination

HRP horse radish peroxidase

HSP70 heat shock protein 70

IP immunoprecipitation

IPTG isopropyl β-D-thiogalactopyranoside

IR ionizing radiation

KARP-1 Ku86 Autoantigen Related Protein-1

kDa kiloDaltons

kV kilovolts

lacZ β-galactosidase

LB Leuria Bertani (medium)

LiAc lithium acetate

Lif1 ligase interacting factor 1

LMB leptomycine B

LTR long terminal repeat

LYS2 lysine 2

mAb monoclonal antibody

MAR matrix attachment region

MCS multiple cloning site

min minutes

ml millilitre

mM millimolar

MMTV mouse mammary tumour virus

Mre11 meitotic recombination 11

mRNA messenger RNA

N nuclear

NES nuclear export signal

NHEJ nonhomologous end joining

NHR nonhomologous recombination

NL nuclear localization

NLS nuclear localization signal

NLS⁻ nuclear localization signal deficient

NP nuclear pore

NP-40 Nonidet P-40

NPC nuclear pore complex

NRE1 negative regulatory element 1

N-terminal amino terminal

NTF2 nuclear transport factor 2

Oct-1 octamer 1

Oct-2 octamer 2

OD optical density

O/N overnight

PAF400 PCAF-associated factor 400

PBS phosphate buffered saline

PCR polymerase chain reaction

PEG polyethylene glycol

PGK phosphoglycerate kinase

PMSF phenylmethylsulfonyl fluoride

Pol I, II RNA polymerase I, II

Poly (dA-dT) poly(deoxyadenylic acid-deoxythymidylic acid)

PP2Ac protein phosphatase 2A catalytic subunit

PVDF polyvinylidene fluoride

RAG1 recombination activating gene 1

RAG2 recombination activating gene 2

RanBP Ran binding protein

RanGAP1 Ran GTPase activating protein

RCC1 regulator of chromosome condensation 1

REF1 redox exchange factor 1

RNA ribonucleic acid

rpm revolutions per minute

RT room temperature

s seconds

S svedberg

SAP SAF-A/B, Acinus, PIAS

SAR scaffold attachment region

S. cerevisiae *Saccharomyces cerevisiae*

SCID severe combined immunodeficiency

SD synthetic dropout

SDS sodium dodecyl sulphate

SDS-PAGE SDS polyacrylamide gel electrophoresis

Sir silent information regulator

SL1 selectivity factor 1

SLE systemic lupus erythematosus

Srp1p serine-rich RNA polymerase I suppressor protein

SSDNA salmon sperm DNA

SV40 simian virus 40

TBP TATA box binding protein

TBS tris buffered saline

TE tris/EDTA

TEMED N, N, N′, N′-tetramethylethyenediamine

TCR T-cell receptor

TFIIB transcription factor IIB

TPE telomere position effect

TRRAP transformation/transcription domain associated protein

TRF2 telomeric repeat binding factor 2

TS temperature sensitive

UAS upstream activating sequence

URA3 uracil 3

UV ultraviolet

V volts

V(D)J variable (diversity) joining

vol volume

v/v volume per volume

WCE whole cell extract

WT wild-type

w/v weight per volume

Xgal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

XRCC x-ray cross complementation

YPD yeast/peptone/dextrose

General Introduction

In higher eukaryotes, the well-being of the organism is dependent on the coordinate interaction of many different tissues. Each tissue is composed of specialized cells that work together to determine the function of the tissue. Much like cells of different tissues perform specialized functions, within the cell distinct compartments exist that are designed for specific cellular processes. Appropriate interactions between numerous proteins, nucleic acids, lipids etc. is required for the viability not only of the cell but the tissue and organism. Therefore, cells have evolved mechanisms to distribute the necessary molecules to the appropriate subcellular compartments. The distribution of a protein to a specific area of the cell may ultimately determine its function. Also, the functions of some proteins are regulated by interactions with other proteins or by environmental stimuli. The Ku antigen is a protein that has been implicated in many distinct cellular processes and Ku function depends on its subcellular distribution and interaction with other proteins. Further, Ku is important for the cell to respond appropriately to certain environmental stresses, namely, those inflicting DNA damage.

Ku Antigen

Ku antigen is an abundant, DNA binding phosphoprotein composed of two polypeptides of 70 and 80 kDa (Ku70 and Ku80, respectively) that associate tightly *in vivo*. It has been estimated that 4 X 10^5 Ku molecules are present per HeLa cell nucleus (Francoeur et al., 1986; Mimori et al., 1981). Ku is a mostly nuclear protein that is

broadly expressed and is highly conserved between eukaryotes. Ku was first identified as an antigen recognized by the serum of patients with autoimmune diseases (Francoeur et al., 1986; Mimori et al., 1981; Reeves, 1985). Anti-Ku antibodies have been identified in patients suffering from systemic lupus erythematosus (SLE), myositis, rheumatoid arthritis, graves disease and Sjogren's syndrome (Arnett et al., 1996; Birdi et al., 1993; Maddison, 1991; Yamanishi et al., 1996; Yaneva and Arnett, 1989). Ku is a highly immunogenic protein with autoantibodies having been mapped almost across the entire amino acid sequence of both subunits. However, a disproportionately high number of autoantibodies recognize the extreme C-terminal domain of Ku70 (Reeves, 1992; Reeves et al., 1991). To date, Ku antibodies are strongly correlated with Raynaud's phenomena and studies have reported Ku antibodies in up to 55 % of patients diagnosed with overlap and SLE (Francoeur et al., 1986; Mimori, 1987; Reeves, 1985; Reeves, 1992; Yaneva and Arnett, 1989). In some of these patients Ku antibody titers can reach 1.5×10^{-7} or higher (Reeves, 1985).

Patients with mixed connective tissue diseases show abnormalities in the tissues that form the framework that holds the cells in the body together, namely collagen and elastin. Marfan syndrome and Ehlers-Danlos syndrome are mixed connective tissue diseases that are strictly inheritable. By contrast, SLE, rheumatoid arthritis, scleroderma, dermatomyositis and polymyositis are the classical connective tissue diseases that do not have specific gene abnormalities as their only cause. These diseases are characterized by the presence of unusual antibodies in the blood. Typically, these patients display high quantities of autoantibodies to nuclear proteins. To date, the mechanism by which many of these patients develop antibodies specific for Ku is not understood. Whether the anti-

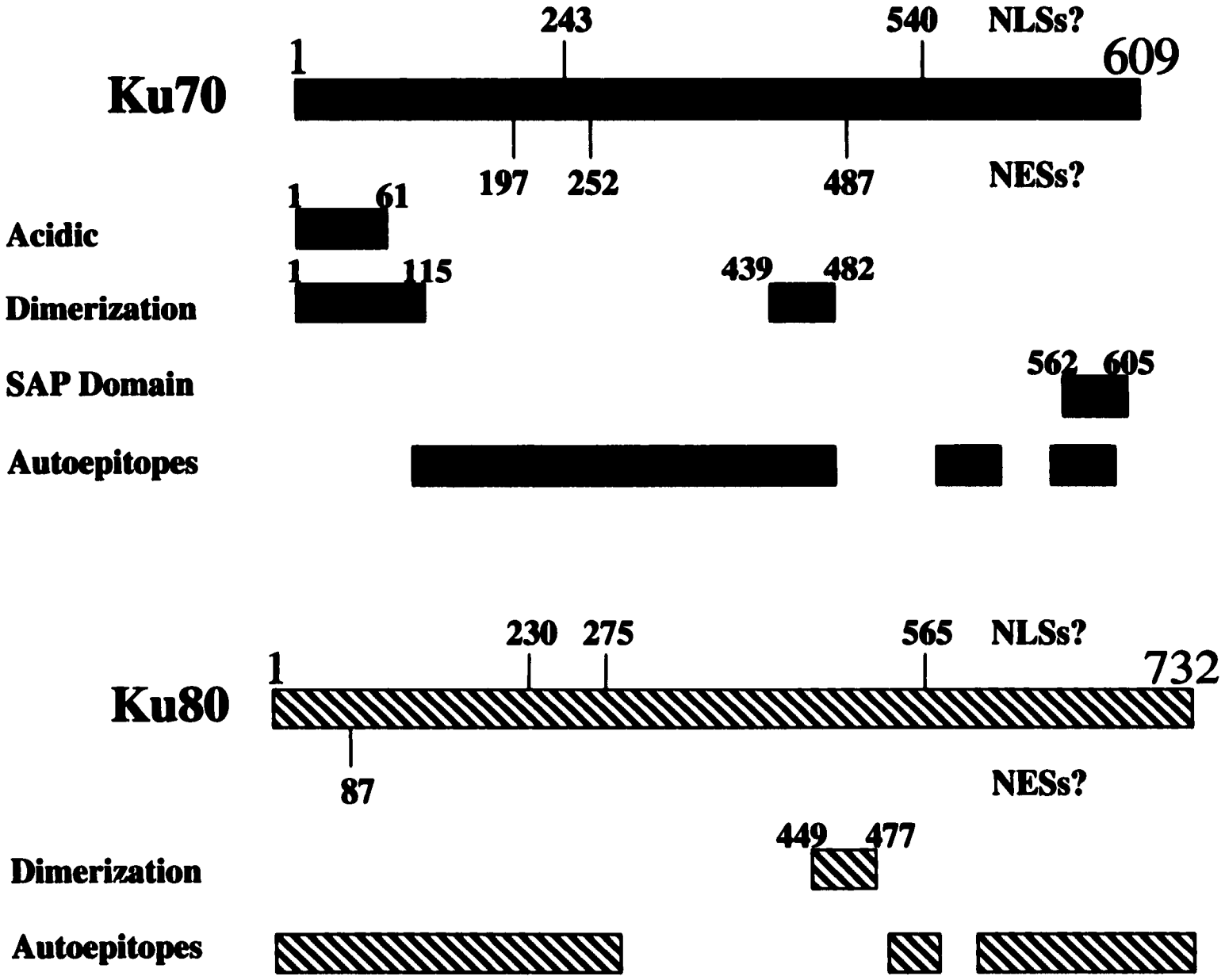
Ku antibodies in many patients with mixed connective tissue diseases arise as a consequence of the disease or whether antibodies to Ku participate in the manifestation of the disease remains to be determined.

A schematic illustration of features within the amino acid sequence of human Ku70 and Ku80 is shown in Figure 1. The Ku70 and Ku80 subunits are composed of 609 and 732 amino acids, respectively. The primary amino acid sequence of both Ku70 and Ku80 is relatively nondescript. However, the extreme N-terminal 6 kDa of Ku70 is highly acidic containing a high proportion of aspartic and glutamic acid residues. Also, the C-terminus of Ku70 contains a 5 kDa region that resembles a SAP (SAF-A/B, Acinus, PIAS) domain (Aravind and Koonin, 2000). The SAP domain closely resembles sequences found in proteins that bind AT-rich chromosomal DNA regions known as scaffold- or matrix-attachment regions (SAR/MAR). Although this is a DNA binding domain it is unrelated to any other known DNA-binding motifs and the role that this region plays in Ku binding to DNA is unknown.

Regions of the Ku subunits required for heterodimerization have now been proposed based on biochemical studies. The proposed dimerization interface of Ku70 spans amino acids 439-482 (Jin and Weaver, 1997; Wang et al., 1998a). An extreme N-terminal region of Ku70 encompassing amino acids 1-115 has also been shown to interact with Ku80 although this region interacts more weakly (Wang et al., 1998a). For Ku80, amino acids 449-477 have been identified to mediate dimerization with Ku70 (Cary et al., 1998; Osipovich et al., 1997; Wang et al., 1998b; Wu and Lieber, 1996).

In addition to the acidic and SAP domains of Ku70, the amino acid sequence of Ku70 exhibits several peaks of hydophobicity and at least one region is of sufficient

Figure 1. Schematic illustration of features within the human Ku Antigen amino acid sequence. Features of Ku70 (top) and Ku80 (bottom) are indicated to the left and numbered bars represent their positions within the amino acid sequences. Location of putative nuclear localization signals (NLSs) and nuclear export signals (NESs) determined by sequence homology is shown.


Ku70
1
243
540
NLSs?
609
197
252
487
NESs?
1
61
Acidic
1
115
439
482
Dimerization
562
605
SAP Domain
Autoepitopes
Ku80
1
230
275
565
NLSs?
732
87
NESs?
449
477
Dimerization
Autoepitopes

length to qualify as a membrane-spanning element (Prabhakar et al., 1990). Also, sequence alignment of Ku subunits between different species reveal sequence homology that spans almost the entire amino acid sequence of both subunits. Notably however, the C-terminal SAP domain of Ku70 is poorly conserved in the yeast homologue.

A phenomenon of Ku is the relative instability of the individual Ku subunits and the requirement for each Ku monomer for stabilization of its partner. The necessity for the presence of both subunits in order to achieve high levels of Ku in the cell is underscored by the low levels of Ku70 in Ku80 deficient cells and the almost undetectable levels of Ku80 in cells lacking Ku70 (Boubnov et al., 1995; Gu et al., 1997a; Nussenzweig et al., 1996). The dramatic increase in stability of the Ku heterodimer compared to the individual Ku monomers despite no obvious change in gene expression suggests that dimerization of the subunits greatly decreases the degradation of the monomers. Whether this is accomplished by the masking of putative degradation signals in the monomers by the other Ku subunit in the context of the heterodimer or whether the increased stability is simply due to a conformational change of the individual subunits upon assembling into a heterodimer needs further investigation.

Both Ku70 and Ku80 are ubiquitously expressed. However, in rodents, higher levels of Ku were detected in the brain and testis compared to the lungs, kidneys and liver (Bakalkin et al., 1998b). Further, embryonic rat brain contains more Ku than adult brain possibly indicating an increased demand for Ku activity in embryogenesis (Bakalkin et al., 1998b).

For Ku80, a variant form of the protein (KARP-1) has been reported that results in an extra 9 kDa of protein appended to the N-terminus of the normal Ku80 (Myung et

al., 1997). The KARP-1 gene has been proposed to use an upstream promoter and additional exons (Myung et al., 1997). However, the biological significance of this form of Ku80, in which expression seems to be restricted to primates, still needs to be confirmed.

Ku Regulates DNA-PKcs Function

In many of the cellular processes in which Ku functions it does so as a regulatory subunit of the DNA-dependent protein kinase (DNA-PK). DNA-PK is composed of the Ku heterodimer and a 450 kDa catalytic subunit (DNA-PKcs) (Gottlieb and Jackson, 1993). DNA-PK is a serine/threonine protein kinase that has been shown to phosphorylate a broad spectrum of proteins *in vitro* including Ku70, Ku80, Oct-1, Oct-2, Sp1, p53 and others (Jackson et al., 1990; Lees-Miller et al., 1990; Lees-Miller et al., 1992; Wang and Eckhart, 1992). DNA-PK is a member of a family of large phosphatidylinositol 3-kinase-related kinases that also includes the ataxia-telangiectasia-mutated (ATM) gene product, PAF400/TRRAP and ATR (Cimprich et al., 1996; Hartley et al., 1995; McMahon et al., 2000; Rotman and Shiloh, 1999; Saleh et al., 1998; Smith and Jackson, 1999; Zakian, 1995). Ku/DNA-PKcs function together in double-stranded DNA break repair, V(D)J recombination and the regulation of mouse mammary tumour virus (MMTV) transcription (Blunt et al., 1995a; Giffin et al., 1996; Kirchgessner et al., 1995; Peterson et al., 1995).

Mice engineered to lack DNA-PKcs display a severe combined immunodeficient (SCID) phenotype and are defective in DNA double-stranded break (DSB) repair

consistent with their hypersensitivity to ionizing radiation. Ku is both a DNA binding subunit and an allosteric activator of DNA-PKcs (Dvir et al., 1992; Giffin et al., 1997; Giffin et al., 1996; Gottlieb and Jackson, 1993; Lees-Miller, 1996; Suwa et al., 1994). Ku bound to DNA ends, structured single-stranded DNA, covalently closed hairpins and direct Ku binding sequences can recruit the catalytic subunit and stimulate kinase activity *in vitro* (Giffin et al., 1997; Hammarsten et al., 2000; Soubeyrand et al., 2001; Yaneva et al., 1997).

Ku is a Multifaceted DNA Binding Protein

Many of the functions ascribed to Ku depend largely on its ability to bind DNA. Ku is probably the most versatile DNA binding protein identified to date. Ku binds with high affinity to DNA ends, nicks, gaps, and virtually every DNA structure that includes a double-to-single-standed DNA transition (Blier et al., 1993; Falzon et al., 1993; Mimori and Hardin, 1986; Paillard and Strauss, 1991; Tuteja et al., 1994). However, Ku does not bind to covalently closed DNA circles and binds only weakly to single-stranded DNA (Griffith et al., 1992; Mimori and Hardin, 1986; Tuteja et al., 1994). Ku is also an enzyme with ATPase (Cao et al., 1994) as well as DNA helicase activity (Ochem et al., 1997; Tuteja et al., 1994). The Ku helicase activity appears to be limited to the unwinding of linear double-stranded DNAs with extended single-stranded overhangs. Moreover, Ku displays no helicase activity from DNA nicks (Tuteja et al., 1994). Also, the helicase activity has been reported to proceed from the 3' to 5' direction (Tuteja et al., 1994).

Once bound to DNA, Ku has the ability to translocate linearly across double-stranded DNA templates from DNA ends and specific sequences in a process that seems to be facilitated by the presence of magnesium and appears to be energy independent (Blier et al., 1993; de Vries et al., 1989; Paillard and Strauss, 1991). Ku has been footprinted over several DNA sequences. Since many of these sequences are not directly recognized by Ku, translocation of Ku along the DNA template and pausing at these sites has been proposed as the mechanism by which Ku accumulates over these sequences (Giffin et al., 1997; Giffin et al., 1996).

Ku also has the ability to transfer between DNA molecules with homologous and nonhomologous DNA ends (Bliss and Lane, 1997; Chiu et al., 2001). Consistent with Ku proteins being able to travel along linear DNA, multiple Ku's on a DNA fragment were visualized by electron microscopy and it was reported that each Ku molecule contacts approximately 13-21 base pairs (bps) and successive molecules are aligned at about 25-30 bp intervals (de Vries et al., 1989). This Ku/DNA complex is thought to resemble beads on a string (de Vries et al., 1989).

In addition to DNA structures, our lab has shown that Ku can also bind directly to a specific sequence (Giffin et al., 1996). We identified a 23 bp polypurine/polypyrimidine rich sequence found in the long terminal repeat (LTR) of the MMTV that we determined to be a specific and direct binding site for Ku *in vitro* (Giffin et al., 1996). We demonstrated that this sequence was essential for Ku/DNA-PKcs-dependent repression of MMTV in transient transfection assays and termed the sequence negative regulatory element 1 (NRE1) (Giffin et al., 1999; Giffin et al., 1996). Binding of Ku to NRE1 was shown to be sufficient to induce a structural transition in the flanking

DNA sequences and this structural transition in the DNA appears to be necessary for activation of DNA-PK and repression of MMTV (Giffin et al., 1999).

Furthermore, our lab investigated the kinetics of Ku binding to NRE1 and found that Ku binds with higher affinity to NRE1 than to DNA ends (Giffin et al., 1994). Moreover, we demonstrated that recombinant Ku bound the single, upper strand of NRE1 with an affinity that was about 3 to 4-fold lower than its binding affinity for double-stranded NRE1 (Torrance et al., 1998). Also, the sequence-specific binding to the single-strand was rapid and stable with an on rate of $t_{1/2}$= 2.0 minutes and an off rate of $t_{1/2}$= 68 minutes (Torrance et al., 1998). Ku70 was shown to cross-link to the upper strand of NRE1 when Ku was bound to double or single-stranded DNAs (Torrance et al., 1998). The Ku80 subunit only cross-linked to the upper single-stranded NRE1, however, the addition of Mg^{2+} and ATP, the co-factors required for Ku helicase activity, promoted the ultra violet (UV) cross-linking of Ku80 to the upper strand of double-stranded NRE1 without completely unwinding the two strands (Torrance et al., 1998). We have also shown that Ku is a flexible protein that can adopt several DNA-dependent conformations that differentially control the recruitment and activation of DNA-PKcs (Giffin et al., 1999).

In addition to NRE1, other sequences have been proposed as specific Ku binding sites. Ku has been shown to interact with origins of replication (Novac et al., 2001; Ruiz et al., 1999b). The A3/4 sequence found in various mammalian replication origins has been proposed as a binding site for Ku (Ruiz et al., 1999a). In this instance, it appears that the DNA contacts of the Ku heterodimer are mainly through the Ku80 subunit as demonstrated by Southwestern analysis (Ruiz et al., 1999b). Further, Ku has been

proposed to interact with other sequences thought to regulate transcription of specifc genes. Ku interaction with these sequences will be discussed below.

Recently, the crystal structure of the Ku heterodimer bound to a DNA end comprised of the full-length Ku70 and a truncated Ku80 that lacks 19 kDa of the C-terminus that was previously shown to function in the recruitment of DNA-PKcs was reported (Walker et al., 2001). The Ku heterodimer has overall dimensions of approximately 120X70X60 Å. In the context of the Ku heterodimer both subunits share a common topology and together form a quasi-symetrical molecule. The close resemblance in structure of the two subunits indicates that the Ku heterodimer most likely evolved from a homodimer. The amino acids that form the dimerization interface share a relatively low sequence identity that may function to exclude the formation of Ku70 and Ku80 homodimers. Both Ku subunits share a three-domain topology. There is an amino-terminal α/β domain, a central β-barrel domain and a helical C-terminal arm. The Ku heterodimer is a dyad-symetrical molecule with a ring structure that can encircle double-stranded DNA ends. This DNA binding site can cradle two full turns of DNA while only encircling the central 3-4 bps.

For detection of high affinity binding of Ku to DNA ends using electrophoretic mobility shift assays (EMSA) both Ku subunits must be appropriately assembled into a heterodimer consistent with both subunits of Ku participating to form a ring structure that binds the DNA end. However, several reports have demonstrated that under less stringent *in vitro* conditions such as DNA immunoprecipitation assays and Southwestern analysis the Ku70 subunit can bind to DNA in the absence of Ku80 (Allaway et al., 1990; Chou et al., 1992; Mimori and Hardin, 1986; Wang et al., 1998a).

Dissection of the determinants that are required for DNA binding by the Ku70 monomer revealed that the C-terminus of Ku70 binds DNA (Chou et al., 1992; Wang et al., 1998a). The N-terminus of Ku70 representing amino acids 1-115 was shown to bind poorly to DNA on its own but upon assembly with Ku80 the DNA binding ability of the resulting heterodimer increased (Wang et al., 1998a). Further, dimerization of a Ku70 mutant lacking amino acids 543-609 from its C-terminus with Ku80 also increased the affinity of this mutant for DNA (Wang et al., 1998a). It therefore would appear that Ku has different modes of DNA binding one of which depends mostly on the Ku70 subunit. Modes in which Ku binds to DNA may be further dependent on the structure of the DNA that Ku binds.

Studies analyzing Ku binding to DNA ends revealed that Ku binding to DNA varies in certain cancers (Pucci et al., 2001). Tumour specific modulations in the ability of Ku to bind DNA were reported in breast and bladder tumour biopsies. In low aggressive breast tumours Ku was expressed in tumour but not in normal tissues and the Ku DNA binding activity correlated with protein levels. In noninvasive bladder carcinomas there was no significant differences in protein level between normal and tumour tissues. However, in this case Ku binding to DNA was increased in the tumour samples. In advanced breast and bladder tumours as well as in node metastases the binding of Ku to DNA was significantly reduced in the tumour samples despite no differences in Ku expression levels. These results indicate that in human neoplastic tissues the binding activities of Ku are possibly modulated by tumour progression.

Ku and the Regulation of Gene Transcription

A role for Ku in the regulation of gene transcription is now well established (Boulton and Jackson, 1998; Dvir et al., 1993; Eggleston, 2000; Evans et al., 1998; Giffin et al., 1999; Giffin et al., 1996; Kuhn et al., 1995; Mishra and Shore, 1999). A few reports have presented data that suggests Ku might play a general role in gene transcription. Ku has been proposed to function in the regulation of both RNA polymerase I and II (Pol-I, II) transcription (Dvir et al., 1992; Labhart, 1995). In addition, Ku has been shown to co-fractionate with the RNA polymerase II holoenzyme and phosphorylation of TATA box binding protein (TBP) and transcription factor II B (TFIIB) by Ku/DNA-PKcs was reported to synergistically stimulate Pol-II basal transcription *in vitro* (Chibazakura et al., 1997; Dvir et al., 1992). Also, there is strong evidence that in yeast Ku in association with Sir proteins functions to repress genes located near telomeres in a process known as the telomere position effect (TPE) (Boulton and Jackson, 1998; Eggleston, 2000; Evans et al., 1998; Mishra and Shore, 1999).

Alternatively, Ku has been proposed to regulate transcription of specific genes. We have shown that Ku/DNA-PKcs is required for repression of steroid induced viral transcription of MMTV through the direct Ku binding site, NRE1 (Giffin et al., 1996). Moreover, in rodent cells overexpression of Ku was demonstrated to repress transcription of the HSP70 gene (Li et al., 1995). Similarly, overexpression of Ku in K562 cells resulted in the repression of a reporter gene under the control of the glycophorin B (GPB) promoter (Camara-Clayette et al., 1999). In this instance, the Ku-dependent repression was determined to be dependent on an interaction of Ku with the WGATAR motif

positioned at –75 in the GPB promoter. Interestingly, in these overexpression experiments the Ku70 subunit seems to be able to function independently of Ku80.

Ku70 alone was also reported to be sufficient for binding to the E3 motif of the TCR β enhancer implicating Ku70 in the regulation of the T-cell receptor β-chain gene (Messier et al., 1993). Ku was also identified as the factor that binds to a DNA region between 101 to –77 of the human COL3A1 promoter suggesting Ku may be a potential regulator of the collagen III gene (Giampuzzi et al., 2000). Another report demonstrated that Ku forms a complex with TBP on the 'CTC box' that is a common promoter element of the human collagen type IV genes (Genersch et al., 1995). However, how Ku regulates the transcription of these genes and recognizes these sequences that share little sequence homology needs further investigation.

Ku Functions Through the NHEJ DNA Repair Pathway

Various types of DNA lesions can occur naturally during the life of a cell or be induced by DNA damaging agents such as ionizing radiation. Probably the most lethal type of DNA damage is the DSB. Repair of the DNA DSB can occur through the homologous end joining (HEJ) or the nonhomologous end joining (NHEJ) pathways. The HEJ pathway requires extensive homology between DNA molecules to repair the DNA whereas NHEJ requires little or no DNA sequence homology between the recombining DNA fragments (Chu, 1997; Critchlow and Jackson, 1998). In mammals and higher eukaryotes NHEJ is the predominant DNA repair pathway. By contrast, lower eukaryotes such as yeast repair DNA predominately through homologous recombination (HR). However, although mammalian cells predominately repair DSBs by the NHEJ

pathway, mammalian cells with mutations in genes that effect HEJ show pronounced defects in DNA repair and in some cases are nonviable (Takata et al., 1998; Tsuzuki et al., 1996; Xiao and Weaver, 1997; Yamaguchi-Iwai et al., 1999).

Several proteins have been implicated to participate in HEJ. These include Rad51, Mre11, RAD54, RAD51B/C/D, XRCC2 and XRCC3 (Griffin et al., 2000; Liu et al., 1998; Takata et al., 2000; Takata et al., 1998; Takata et al., 2001). In vertebrate cells HR seems to be suppressed in the $G_{0/1}$ phase of the cell cycle. Interestingly, during this phase of the cell cycle the NHEJ pathway seems to be more active. The inactivity of HR during $G_{0/1}$ may avoid competition between factors of the HR and NHR pathways to reach the DNA DSB (Van Dyck et al., 1999).

Ku70 and Ku80 are key components of the NHEJ pathway. Cells with mutations that eliminate the expression of either Ku70 or Ku80 are dramatically sensitive to DNA damaging agents that cause DNA DSBs (Gu et al., 1997a; Taccioli et al., 1994a). In addition to the Ku proteins, DNA ligase IV (Grawunder et al., 1998), XRCC4 (Nick McElhinny et al., 2000) and DNA-PKcs (Smith and Jackson, 1999) have also been implicated in NHEJ. Similar to cells lacking Ku, cells with mutations that eliminate expression of any of these factors, display pronounced defects in DNA DSB repair.

Role of Ku/DNA-PKcs in DNA Repair

The involvement of Ku/DNA-PKcs in DNA repair was discovered by analysis of mutant rodent cell lines that were defective in DNA DSB repair and showed a heightened sensitivity to ionizing radiation (Gu et al., 1997a; Taccioli et al., 1994b). These mutant

cell lines were also determined to be deficient in immunoglobulin V(D)J recombination. These cells were grouped into three different cross complementation groups IR4, IR5 and IR7 (for ionizing radiation group 4, 5, 7) with their complementing genes called XRCC5, XRCC6 and XRCC7 (for X-ray cross-complementing), respectively. It was demonstrated that IR5 cells in addition to displaying a hypersensitivity to ionizing radiation also lacked a Ku-like DNA end binding activity (Rathmell and Chu, 1994; Smider et al., 1994; Taccioli et al., 1994b). It was demonstrated that ectopic expression of Ku80 complemented the DNA break repair phenotype and restored the Ku-like DNA binding activity. Further studies with IR7 cells revealed that these cells could be complemented with the expression of the DNA-PKcs gene (Kirchgessner et al., 1995). Other experiment confirmed that IR5 and IR7 cells had inactivating mutations in the Ku80 and DNA-PKcs genes, respectively (Blunt et al., 1995b; Danska et al., 1994; Errami et al., 1996). Later, disruption of the gene encoding for Ku70 showed similar defects in DNA repair and these cells formed the IR6 group (Gu et al., 1997a). IR4 cells were shown to be deficient in the XRCC4 gene product. The functions and biochemical properties of this protein are still not well understood, however XRCC4 has been shown to interact with DNA ligase IV and stimulate its DNA end joining activity (Leber et al., 1998).

It is well established that both Ku and DNA-PKcs are necessary for proper functioning of the NHEJ pathway. However, the mechanism by which Ku/DNA-PKcs function in this pathway is poorly understood. There is evidence to suggest Ku/DNA-PKcs may bind directly to DSBs and protect the DNA ends from nucleolytic degradation (de Vries et al., 1989; Gottlieb and Jackson, 1993; Mimori and Hardin, 1986). There is

also data to suggest Ku/DNA-PKcs may bind to two broken ends and tether them together and with the aid of other factors implicated in DSB repair such as XRCC4 and DNA ligase IV join the two ends together (Ramsden and Gellert, 1998). This mechanism is consistent with Ku's ability to juxtapose double-stranded ends *in vitro* (Cary et al., 1997). Recently, it was shown that Rad50, Mre11 and Xrs2 proteins promote DNA end joining by DNA ligase IV and Lif1 *in vitro* and this DNA joining is further stimulated by the addition of yeast Ku (Chen et al., 2001).

Ku is Required for V(D)J Recombination

Ku/DNA-PKcs is required for efficient recombination of immunoglobulin genes. V(D)J recombination results from the rearrangement of immunoglobulin and T-cell receptor (TCR) genes leading to the formation of coding and signal joints (Gellert, 1997). Coding joints encode the highly variable antigen binding regions of the antigen receptors and signal joints are a byproduct of this process. Initially, RAG1 and RAG2 perform the cleavage of the DNA between the signal and coding sequences (Ramsden et al., 1997). This process leads to the formation of blunt ended signal ends and covalently closed hairpin coding termini (Ramsden et al., 1997). Signal ends are ligated by direct end joining, whereas for coding termini the hairpins must be opened prior to ligation (Ramsden et al., 1997).

In SCID mice, which are severely defective in DNA-PK activity due to a C-terminal truncation of the last 83 amino acids of DNA-PKcs, the formation of coding joints is greatly impaired while signal joints are formed albeit with reduced efficiency

(Blunt et al., 1996; Bogue et al., 1998; Danska et al., 1994). Mice lacking DNA-PKcs also display this same phenotype (Jhappan et al., 1997; Taccioli et al., 1998). By contrast, mice generated to lack Ku70 or Ku80 display defects in both signal and coding joint formation (Zhu et al., 1996). However, for Ku70 deficient mice residual amounts of signal joints are observed (Gu et al., 1997b). Together, these results suggest that DNA-PKcs may play a more direct role in resolving hairpin ends of coding joints and Ku is required for formation of both signal and coding joints. Consistent with a role for Ku/DNA-PKcs in resolving the hairpin structure of coding joints was the recent observations our lab made that DNA-PK shows kinase activity from covalently closed DNA hairpin ends (Soubeyrand et al., 2001).

A Role for Ku in Telomere Maintenance, Cell Cycle Control, DNA Replication, Neuronal Survival, Growth and Tumour Suppression

Ku functions in numerous cellular processes predominately in the metabolism of DNA. As mentioned above, Ku plays a critical role in DNA DSB repair through the NHEJ pathway and is required for immunoglobulin V(D)J recombination (Finnie et al., 1995; Gu et al., 1997a; Nussenzweig et al., 1996; Nussenzweig et al., 1997; Smider et al., 1994; Taccioli et al., 1994a).

In addition, Ku has been proposed to play an important role in telomere maintenance and protecting telomeres from nucleolytic attack (Boulton and Jackson, 1996; Boulton and Jackson, 1998; Driller et al., 2000; Gravel et al., 1998). A role for Ku in telomere maintenance is supported by the observation that Ku deficient yeast have

shorter telomeres (Boulton and Jackson, 1996). In yeast the Ku heterodimer is associated with both the chromosomal termini and the subtelomeric chromatin (Martin et al., 1999). It has been reported that Ku is relocalized from the telomeric regions in response to DNA damage and recruited to DNA DSBs (Martin et al., 1999). Also in yeast, Ku has been proposed to regulate telomerase function through an interaction with a stem-loop of the telomerase RNA gene (Peterson et al., 2001). Further, Ku seems to be required for telomeric association with the nuclear periphery (Laroche et al., 1998).

Ku has also been shown to associate with telomeres in higher eukaryotes (Bailey et al., 1999; Hsu et al., 1999). *In vivo* cross-linking experiments with mammalian cells have shown that human telomeric DNAs specifically co-immunoprecipitate with Ku80 (Hsu et al., 1999). In a separate report Ku was shown to function in capping mammalian chromosomes and preventing their end-to-end fusion (Bailey et al., 1999).

Recently, Ku80 has been characterized as a caretaker gene that maintains the integrity of the genome by suppressing chromosomal rearrangements (Difilippantonio et al., 2000). In addition, Ku has been proposed to function in controlling the progression of cells through the cell cycle and regulate DNA replication (Lee et al., 1998; Munoz et al., 1998; Novac et al., 2001; Ruiz et al., 1999b; Tuteja and Tuteja, 2000). Consistent with Ku involvement in cell cycle control is the observation that cells deficient in Ku80 show an extended G_2/M transition (Lee et al., 1998). Whether this is a direct result of a deficiency in DNA repair in these cells that lack Ku activity or whether it reflects the participation of Ku in another cellular process distinct from DNA repair needs to be determined. Interestingly, rodent cells lacking Ku80 are more sensitive to the topoisomerase II inhibitor, ICRF-193, than wild-type (WT) cells (Munoz et al., 1998).

Ku80 deficient cells were shown to accumulate at the G_2/M stage of the cell cycle upon treatment of the cells with a low dose of ICRF-193 (Munoz et al., 1998). The lack of detectable DNA DSBs upon treatment of the cells with this topoisomerase II inhibitor and the finding that cells deficient in DNA-PK activity do not accumulate in G_2/M following inhibitor treatment suggests that the arrest at the G_2/M phase of the cell cycle may result from a defect in a function of Ku separate from DNA repair.

Moreover, Ku plays a critical role in neurogenesis (Gu et al., 2000). Mice deficient in Ku70 and Ku80 have a dramatic increase of death in developing embryonic neurons (Gu et al., 2000). A similar phenotype was observed with XRCC4 and DNA ligase IV deficient mice, however the phenotype in these mice was significantly more pronounced than for mice lacking Ku (Frank et al., 2000; Sekiguchi et al., 1999). Interestingly, mice lacking DNA-PKcs did not share this phenotype (Gu et al., 2000).

Mice deficient in Ku70 or Ku80 share many overlapping phenotypes. Whether this overlap in the phenotypes reflect a requirement for the Ku heterodimer or the low levels of the Ku monomers in the absence of their partner is not clear. Ku70 and Ku80 deficient mice are severely immunocompromised and are hypersensitive to ionizing radiation as well as to other agents that induce DNA DSBs (Gu et al., 1997a; Nussenzweig et al., 1996; Ouyang et al., 1997; Zhu et al., 1996). Ku knockout mice age prematurely and display genomic instability (Vogel et al., 1999). A characteristic phenotype of mice lacking Ku is that they are proportional dwarfs (Gu et al., 1997c; Nussenzweig et al., 1996). Both $Ku70^{-/-}$ and $Ku80^{-/-}$ mice are approximately half the size of their WT littermates. Consistent with this growth retardation is the observation that $Ku^{-/-}$ fibroblasts grow more slowly in culture. Furthermore, this growth deficiency

appears to be conserved from yeast to humans as yeast deficient in the Ku70 homologue (HDF1) or the Ku80 homologue (HDF2) also grow more slowly at 30 °C (Barnes and Rio, 1997; Boulton and Jackson, 1996). In addition, Ku deficient yeast display a temperature sensitive (TS) phenotype as they die when incubated at 37 °C whereas the parent strain grows normally (Barnes and Rio, 1997; Boulton and Jackson, 1996). At least for HDF1 deficient yeast incubated at 37 °C most of the dead cells are arrested at the G_2/M transition (Barnes and Rio, 1997). Also, mitotically growing yeast deleted for the Ku70 homologue show an abnormally high DNA content that may suggest that in the absence of HDF1 DNA replication is uncoupled from cell division (Barnes and Rio, 1997).

Despite many similarities in mice deficient in either Ku subunit, $Ku70^{-/-}$ and $Ku80^{-/-}$ mice do have specific differences in their phenotypes that may indicate specific roles for the individual Ku subunits in the cell. In $Ku80^{-/-}$ mice, T and B-cell development is arrested at the early, $CD4^-CD8^-$ double negative, $B220^+CD43^+$ progenitor stage (Nussenzweig et al., 1996; Zhu et al., 1996). By contrast, in $Ku70^{-/-}$ mice B-cell development is completely arrested, however T-cells develop to mature $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive cells (Gu et al., 1997a; Ouyang et al., 1997). Although $Ku70^{-/-}$ mice have mature T-cells their numbers are dramatically reduced when compared to their WT littermates. Furthermore, Ku70 but not Ku80 deficient mice develop spontaneous $CD4^+CD8^+$ thymic and disseminated cell lymphomas at approximately 6 months of age suggesting that the Ku70 monomer may have an additional function as a tumour suppressor in thymocytes (Gu et al., 1997b; Li et al., 1998). Furthermore, Ku70 deficient

fibroblasts display an increase rate of sister chromatid exchange and a high frequency of spontaneous neoplastic transformation (Li et al., 1998).

Ku Binding Proteins

Ku has been implicated in many cellular processes and in some, Ku seems to function independently of DNA-PKcs (Gravel et al., 1998; Gu et al., 2000; Laroche et al., 1998). This suggests that Ku may interact functionally with other proteins in the cell. Many proteins have now been identified to interact with Ku.

Ku interacts with several proteins that function in the regulation of gene transcription. Ku70 has been shown to associate with GCN5, a putative transcriptional adapter in humans and yeast that has histone acetyl transferase (HAT) activity (Barlev et al., 1998). Phosphorylation of GCN5 by DNA-PK was shown to inhibit its HAT activity (Barlev et al., 1998). Using EMSA it was shown that Ku bound to the 'CTC box' of the collagen IV genes could form a complex with TBP, a protein required for RNA polymerases I, II and III transcription (Genersch et al., 1995). Our laboratory using the yeast two-hybrid system found that a broad spectrum of homeodomain proteins interact with Ku70 including HOXC4, octamer transcription factors 1 and 2 and Dlx2 (Schild-Poulter et al., 2001). We determined that the homeodomain proteins interact with a basic region in the C-terminus of Ku70 through their homeodomains (Schild-Poulter et al., 2001). The Ku/homeodomain protein interaction was shown to increase phosphorylation of the homeodomain proteins by DNA-PK *in vitro* (Schild-Poulter et al., 2001). In addition, heat shock transcription factor 1 binding to Ku was shown to stimulate DNA-

PK activity *in vitro* (Huang et al., 1997). Ku was also reported to interact with REF1, a redox factor that regulates genes through negative calcium-responsive elements (Chung et al., 1996). Ku binding to REF1 led to a complex with a novel sequence specificity that regulated gene expression (Chung et al., 1996). Moreover, Ku70 was shown to interact with the heterochromatin protein 1α that is a suppressor of the position effect variegation in *Drosophila* and a transcriptional suppressor in mammalian cells (Song et al., 2001).

Ku has also been reported to interact with proteins involved in the maintenance of DNA integrity. Ku70 interacts with Mre11, a protein that functions in both the NHEJ pathway and meiotic HR (Goedecke et al., 1999). In this instance, Ku70 was proposed to function as a molecular switch between the NHEJ pathway and the HR pathway (Goedecke et al., 1999). The yeast-two hybrid system was used to identify TRF2 as a Ku70 binding protein (Song et al., 2000). TRF2 is a mammalian telomere-binding protein. Also, it was reported that recombinant XRCC4 facilitates Ku binding to DNA and promotes assembly of DNA-PK suggesting XRCC4 may function as an alignment factor in the DNA-PK complex (Leber et al., 1998).

Moreover, the hematopoeitic tissue-specific protooncogene p95vav has been reported to bind to Ku70, although the functional relevance of this interaction is still unclear (Adam et al., 2000). Further, Ku80 was reported to bind to protein phosphatase 2A ($PP2A_c$) and repress its catalytic activity (Le Romancer et al., 1994). It was reported that binding of somatostatin to Ku80 antagonized this Ku80-dependent inhibition (Le Romancer et al., 1994). The interaction of Ku with many factors that have been implicated in diverse processes suggests that Ku may have many distinct functions in the

cell. However, the *in vivo* biological significance of many of these interactions mentioned needs confirmation.

Overview of Nucleo-Cytoplasmic Trafficking

The functions of many proteins are regulated by their subcellular localization. The distribution of many proteins within the cell is regulated by the concerted action of a number of soluble nuclear import and nuclear export factors that actively mediate their passage through the nuclear pore complex (NPC). The NPC is a 125-mega Dalton structure that spans the double lipid bilayer of the nuclear envelope and is composed of over 100 different polypeptides termed nucleoporines (Davis, 1995; Doye and Hurt, 1997; Pante and Aebi, 1996). The NPC regulates the nuclear import and export of many proteins by functioning as a gatekeeper allowing entry or exit from the nucleus of almost all proteins larger than 60 kDa in size which cannot passively diffuse through the nuclear pore.

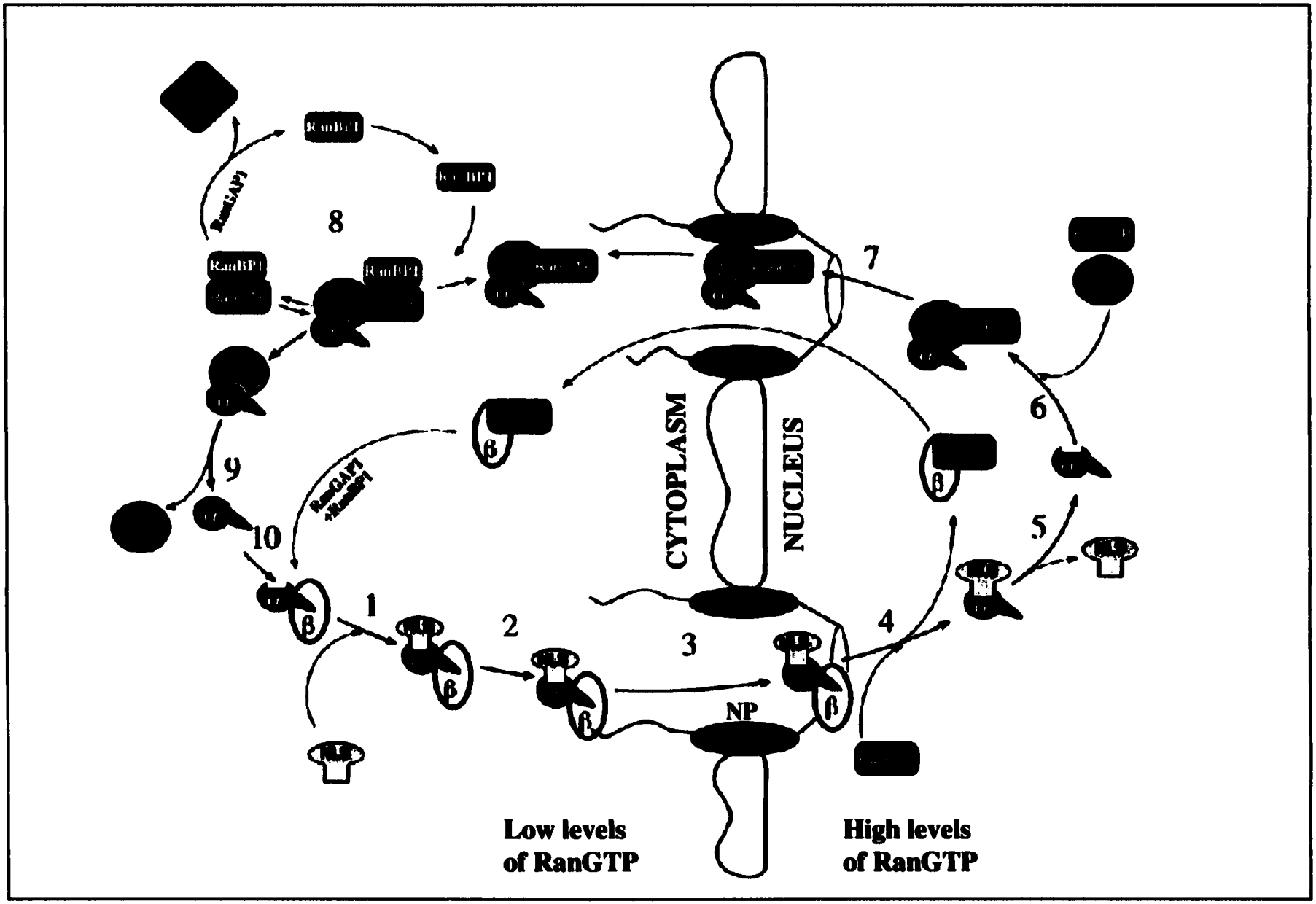
In recent years there has been a virtual explosion in the understanding of the factors that mediate the nucleo-cytoplasmic trafficking of proteins. The first breakthrough came with the identification of a signal that was determined to be required for the nuclear import of the SV40 T antigen (Kalderon et al., 1984; Lanford and Butel, 1984). This was a short sequence enriched in basic amino acid residues (e.g. PKKKRKV) and was named a nuclear localization signal (NLS) (Kalderon et al., 1984; Lanford and Butel, 1984). To date, the NLSs of numerous proteins have been identified. Most of these NLSs can be separated into two categories that represent the classical NLSs (Dingwall and Laskey, 1991). Sequences that contain a single cluster of basic amino acid

residues are termed monopartite and those which are composed of two basic amino acid clusters that are usually separated by a 8-10 amino acid spacer region are called bipartite. Still, other NLSs that do not conform to either of these two types have been identified. One example is the nuclear import signal M9 of the hnRNP A1 that is glycine rich rather than basic (Pollard et al., 1996). In addition, a unique signal was identified for the hnRNP K termed KNS and this signal has been reported to mediate nuclear import independent of any soluble factors (Michael et al., 1997). Also, some nuclear import signals such as the M9 sequence have been shown to mediate both the nuclear import and nuclear export of proteins (Pollard et al., 1996).

Many of the import receptors for proteins bearing the classical NLSs are now known. Importin β is the prototype of a family of nuclear import and export receptors (Fornerod et al., 1997b; Gorlich et al., 1997). Importin β can mediate the nuclear transfer of proteins with basic NLSs either by directly binding to the NLS of the protein or indirectly by associating with the amino terminus of the adapter protein importin α that binds the basic NLS (Gorlich et al., 1996a; Weis et al., 1996). A schematic diagram summarizing the process of nuclear import of proteins bearing classical NLSs and requiring importin α for nuclear uptake is shown in Figure 2. Many of the facets of nucleo-cytoplasmic trafficking have been reviewed (Gorlich, 1998; Hurt, 1996; Ullman et al., 1997).

Importin α directly recognizes the basic cluster that comprises the NLS via a core of eight armadillo repeats (Conti et al., 1998). To date, there have been 6 importin α isoforms identified (Cortes et al., 1994; Cuomo et al., 1994; Kohler et al., 1997; Nachury

Figure 2. Diagram of the importin α/β nuclear import pathway. Proteins larger than 60 kDa in size usually enter or leave the nucleus through a highly regulated process. Proteins to be imported into the nucleus usually contain a nuclear localisation signal that is most often a short basic amino acid sequence. This sequence mediates protein (NLS) binding to the adapter protein, importin α (α), which associates with the nuclear import receptor, importin β (β) (1). Importin α and the protein bearing the nuclear localisation signal are targeted to the nuclear pore (NP) by importin β (2). The complex then translocates through the nuclear pore through a mechanism that is not well understood but appears to require energy (3). Once on the nuclear side the trimeric complex interacts with RanGTP (4) that promotes the dissociation of the subunits and release of the imported cargo (5). The RanGTP/importin β complex is then recycled back to the cytoplasm. Importin α is exported by its export receptor CAS. CAS and importin α associate in the nucleus in a co-operative fashion with RanGTP (6) before docking to the NP and translocation through to the cytoplasm (7). Once in the cytoplasm RanGTP is hydrolysed to RanGDP by the concerted action of specific enzymes including RanBP1 and RanGAP1 (8) promoting the dissociation of the complex and the release of importin α (9). Importin α is then available for another round of nuclear import (10).



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et al., 1998; Seki et al., 1997). Based on their primary amino acid sequence homology the importin α's have been grouped into three subfamilies. Importin α1 is the sole member of one family, importin α3 and 4 make up the second family and importin α 5, 6 and 7 form the third family. Within one family the sequence homology is about 80 % and between subfamilies it is approximately 50 %. In addition, partial cDNAs of importin α6 and 7 have been found in the database that may represent alternatively spliced mRNAs. Although most importin α's are expressed in all tissues, except for importin α6 which has only been found in the testis, their expression levels vary suggesting a particular demand for specific importin α's in different tissues (Kohler et al., 1999).

For many proteins the different isoforms of importin α seem to be able to mediate import of the substrate with only marginal differences in efficiency when only one substrate is presented to the receptor (Kohler et al., 1999). However, if two substrates are offered at the same time the importin α's can show striking differences for some substrates in the specific import efficiencies (Kohler et al., 1999). Moreover, some proteins are efficiently imported by a particular importin α. For example, RCC1 is imported efficiently only by importin α3 (Kohler et al., 1999).

The importin β receptor and the importin α adaptor proteins are by no means the only nuclear import factors. Transportin for instance has been shown to mediate the import of proteins containing the M9 sequence (Pollard et al., 1996).

The asymmetric distribution of RanGTP across the nuclear envelope is critical for nucleo-cytoplasmic trafficking. This is supported by experiments designed to disrupt this equilibrium, which show a complete arrest in the nuclear import and export of proteins

(Izaurralde et al., 1997; Kadowaki et al., 1993; Richards et al., 1997). Ran found in the cytoplasm is maintained mostly in its RanGDP bound state by the action of the GTPase-activating protein RanGAP1 that is localized predominately in the cytoplasm or associated with the cytoplasmic face of the NPC. In the nucleus, Ran is kept mostly in its RanGTP bound state by the Ran exchange factor, RCC1, which is localized to the nucleus. NTF2 is another soluble factor implicated in nucleo-cytoplasmic trafficking and has been shown to function in the nuclear import of Ran (Ribbeck et al., 1998).

Most proteins bearing a classical NLS are thought to be bound by importin α in the cytoplasm. The trimeric importin α/importin β/NLS complex docks to the nuclear pore through interactions of importin β with nucleoporins (Gorlich et al., 1995). Following docking, there is translocation of the complex through the nuclear pore in a process that is still not well understood but appears to require energy (Michael et al., 1995; Newmeyer et al., 1986). Once inside the nucleus importin β binds to RanGTP that is readily available and this leads to the dissociation of the imported complex and release of the imported cargo (Chi et al., 1995; Gorlich et al., 1996b; Rexach and Blobel, 1995).

Importin β is though to remain attached to the nuclear pore and return to the cytoplasm rapidly without the aid of any other soluble factors. Once recycled back to the cytoplasm importin β is then available for another round of import. By contrast, the export of importin α is mediated by its nuclear export factor CAS that binds importin α co-operatively with RanGTP in the nucleus and is then exported to the cytoplasm (Kutay et al., 1997). Once in the cytoplasm the importin α is released from CAS by the concerted action of RanGAP1 and RanBP that convert RanGTP to RanGDP. Once

released in the cytoplasm the importin α, like importin β, is available for another round of import.

Similar to proteins that are actively imported into the nucleus, proteins that are actively exported from the nucleus also contain specific sequences that mediate their export. Like NLSs, these sequences are short but are usually rich in leucine residues rather than basic amino acids (Gerace, 1995). Exportin 1/Crm1 was identified as the export receptor that recognized the leucine rich nuclear export signal (NES) of the HIV-1 Rev protein (e.g. LPPLERLTL) and mediated its export from the nucleus (Fornerod et al., 1997a). Proteins to be exported from the nucleus containing leucine rich NESs are bound by Crm1 and RanGTP co-operatively in the nucleus. Analogous to nuclear import the complex docks to the nuclear pore and translocates through to the cytoplasmic side. Hydrolysis of RanGTP in the cytoplasm results in release of the exported cargo from Crm1. Crm1 is then recycled back to the nucleus where it can mediate another round of export.

Hypotheses

A) Ku Antigen regulates gene expression by interacting directly with specific DNA binding sequences in a chromatin environment.

B) Signals that determine the subcellular distribution of Ku Antigen regulate Ku function in the cell.

Specific Objectives

Many functions of Ku depend on the ability of Ku to interact with DNA. Although the nonspecific binding of Ku to DNA ends has been studied significantly *in vitro*, how Ku interacts with DNA in a chromatin environment is not well understood. Ku has been proposed to bind specifically to a number of DNA sequences, however whether or how Ku directly recognizes these sequences that share little sequence homology is not known. We had previously determined through EMSA using covalently closed DNA micocircles that Ku bound directly and specifically to the NRE1 element of the MMTV *in vitro* and Ku binding to this sequence was required for repression of steroid induced transcription of MMTV (Giffin et al., 1996). Therefore, the first objective of my research was to investigate Ku binding to the NRE1 sequence in the intact cell in the context of chromatin.

Although Ku is predominately a nuclear protein several reports have shown Ku to be localized to other compartments within the cell (Dalziel et al., 1992; Higashiura et al., 1992; Koike et al., 1999a; Teoh et al., 1998; Yaneva and Jhiang, 1991; Yu et al., 1998).

In addition to the nucleus, Ku has been localized to the plasma membrane and cytoplasm (Dalziel et al., 1992; Koike et al., 1999b; Pucci et al., 2001; Teoh et al., 1998; Tovari et al., 1998). Since the functions of many proteins are regulated by signals that localize them to discrete areas of the cell, identification of the signals that mediate their subcellular distribution is necessary to completely understand their function. Characterization of the signals that mediate Ku transfer to the nucleus is an important first step in determining how Ku is targeted to the nucleus. Therefore, the second objective of my research was to characterize the signals required for the nuclear import of Ku.

Materials and Methods

Mammalian Expression Plasmids

Ku expression plasmids pBJ5Ku70 and pBJ5Ku80 are mammalian expression vectors containing the Ku70 and Ku80 cDNAs and are described elsewhere (Smider et al., 1994). For generation of pEGFPKu70, human Ku70 cDNA was cloned in frame into the pEGFP-C1 vector (Clontech). pEGFPKu70$_{220\text{-}609,\ 320\text{-}609,\ 379\text{-}609,\ 485\text{-}609}$ deletion constructs were generated using specific restriction enzymes to delete fragments from the Ku70 cDNA of the pEGFPKu70 and then ligating the vector back together. pEGFPKu70$_{1\text{-}491}$ was cloned by isolating the Ku70 cDNA from pEGFPKu70 with BspE1/StuI and then inserting the fragment into pEGFP-C1. For construction of pEGFPKu80, the Ku80 cDNA was cloned into the EcoR1 site of pEGFP-C1 (Clontech). pEGFPKu80$_{1\text{-}212,\ 1\text{-}374,\ 1\text{-}502}$ deletion constructs were generated by restricting pEGFPKu80$_{A453H,V454H}$ removing the cDNA encoding for the indicated amino acids and religating the vector. pEGFPKu80$_{1\text{-}580}$ was constructed by restricting pEGFPKu80$_{A453H,V454H}$ with BsmB1/BamHI, filling in and then religating the vector. pEGFPKu70$_{537\text{-}557}$ was constructed by insert of an oligonucleotide encoding Ku70 amino acids 537-557 (upper strand: 5′-TCGAATTCTGAAGGGAAAGTTACCAAGAGAAAACACGATAATGAAGGTTCTG GAAGCAAAAGGCCCAAGGTGGGATCCAC-3′, lower strand: 5′-GTGGATCCCACCTTGGGCCTTTTGCTTCCAGAACCTTCATTATCGTGTTTTCTC TTGGTAACTTTCCCTTCAGAATTCGA-3′) into pEGFP-C1.

Several pBJ5Ku70, pEGFPKu70 and pEGFPKu80 constructs were prepared using inverse PCR. Small letters denote mutations resulting in amino acid substitutions in the

Ku proteins. The primer set employed were as follows: pEGFPKu70$_{\Delta 537\text{-}557}$, forward primer 5′-ATGCGGCCGCAGAGTATTCAGAAGAGGAGCTGAAGACCCAC-3′, reverse primer 5′-TTGCGCGGCAGGATTGTAATCTGGTGGGTAAACAAGCTC-3′; pBJ5Ku70$_{K542A,R543A,K544A}$, forward primer 5′-gccgcaCACGATAATGAAGGTTCTGGAAGCAAAAGGCCC-3′, reverse primer 5′-CgcGGTAACTTTCCCTTCAGGATTGTAATCTGGTGG-3′; pBJ5Ku70$_{K553V,R554N,K556A}$, forward primer 5′-aacCCCgcaGTGGAGTATTCAGAAGAGGAGCTGAAGACCCAC-3′, reverse primer 5′-aacGCTTCCAGAACCTTCATTATCGTGTTTTCTCTTGGT-3′; pBJ5Ku70$_{58\text{-}609}$, forward primer 5′-TAATTCTCGAGATGACACCTTTTGACATGAGCATCCAGT-3′, reverse primer 5′-TGTAATATGACTCCCACCCTGACAT-3′; pEGFPKu80$_{A453H,V454H}$, forward primer 5′-CTGAATcaccacGATGCTTTGATTGACTCCATGAGCTTGGCAAAGAAA-3′, reverse primer 5′-CTGTGCCTCGGTGGGAGCATATTTCTTACTGTT-3′; pEGFPKu80$_{K565A,K566A,K568A}$, forward primer 5′-gcATTAgcaACTGAGCAAGGGGGAGCCCACTTCAGCGTCTCC-3′, reverse primer 5′-ggccGCTGTAGGTCCATCTTCATGGTTGTCTTGGAA-3′.

Yeast Expression Plasmids

Gal4DBDKu80$_{1\text{-}432}$ and Gal4DBDKu80$_{339\text{-}732}$ constructs were generated by isolating human Ku80 cDNA fragments from VBB2-Kup80 by restricting with Bsu36I/AflIII and

XmnI/BglII, respectively. Fragments were filled in and cloned in frame into the NdeI site of the pAS1-tet yeast/bacteria shuttle vector. The pG-3Ku80 construct was generated by isolating the full-length Ku80 cDNA from VBB2-Kup80 by restricting with Bsu36I/BglII, filling in and cloning the fragment into the BamHI site of the pG-3 vector. pActIIKu70$_{1-609}$ was constructed by isolating the Ku70 cDNA from VBB2-Kup70tH6 by restricting with AflIII/MscI and cloning the fragment into pActII. pActIIKu70 deletion mutants pActIIKu70$_{1-167}$, pActIIKu70$_{1-433}$, pActIIKu70$_{1-491}$, pActIIKu70$_{\Delta 229-362}$, pActIIKu70$_{\Delta 177-362}$, pActIIKu70$_{\Delta 177-492}$, pActIIKu70$_{\Delta 177-584}$, were generated by restricting the pActIIKu70$_{1-609}$ backbone with convenient restriction enzymes cleaving out the cDNA encoding for the indicated amino acids and ligating the vector back together. pActIIKu70$_{58-609}$ was constructed by isolating the Ku70 cDNA from pAS1-tetKu70 by restricting with HincII/MscI and cloning the fragment into the SmaI site of pActII. pActIIKu70$_{1-99}$ was generated by isolating the Ku70 fragment from pAS1-tetKu70 by restricting with NdeI/DraI and cloning into the pActII vector. The pAS1-tetΔDBDKu70 vector expressing Ku70 was generated by removing the Gal4 DNA binding domain from pAS1-tet and cloning the full-length human Ku70 cDNA into the multiple cloning site. pAS1-tetΔDBDKu70/80 expressing both Ku70 and Ku80 was constructed by cloning a DNA fragment containing the GPB promoter, human Ku80 cDNA and the PGK terminator isolated from pG-3Ku80 at a site downstream of the Ku70 cDNA insertion in the polylinker of pAS1-tetΔDBDKu70. pActIIKu80 expressing full-length Ku80 was generated by cloning the human Ku80 cDNA into pActII. pGAD53m (Clontech) was used to express the mouse p53 fused to the Gal4 activation domain. pActIIHDF1 was constructed by PCR amplifying the full-length *HDF1* coding sequence from yeast

genomic DNA with the primer set: forward primer 5'-ATGGCCATGGAGATGCGGCCAGTCACTAATGCATTTGGCAAT-3', reverse primer 5'-ATTCGGATCCTTATATATTGAATTTCGGCTTTTTATCAAA-3' which contains the NcoI and BamHI restriction sequences (underlined), respectively, to facilitate cloning into pActII. The pAS1-tetRab vector was used to express the Rab protein fused to the Gal4 DNA binding domain.

Bacterial Expression Plasmids

pGex-2TKu70 was constructed by isolating the full-length Ku70 cDNA from pActIIKu70$_{1-609}$ by restricting with Nde1/XhoI, filling in and cloning into pGex-2T at the SmaI site. pGex-2TKu70$_{\Delta 177-492}$ was generated by restricting pGex-2TKu70 with convenient enzymes removing the cDNA encoding for amino acids 177-492 of Ku70 and ligating the vector back together. The pET-30a(+)Ku70 plasmid was constructed by cloning the human Ku70 cDNA into the HindIII site of the pET-30a(+) vector. Clones were verified by DNA sequencing and/or protein expression by Western blot analysis.

Preparation of Plasmid DNA

A saturated overnight (O/N) 500 ml culture of transformed DH5α *Escherichia coli* (*E. coli*) was centrifuged and the bacterial pellet resuspended in 1 ml of 25 mg/ml hen egg white lysozyme in GTE solution (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0) and incubated at RT for 10 min. 10 ml of freshly prepared 0.2 M NaOH/1 % SDS (w/v)

solution was then added to the bacteria and mixed gently and incubated 10 min on ice. 7.5 ml of 3 M potassium acetate solution (3 M potassium acetate, 12 % glacial acetic acid (w/v)) was then added, the solution mixed and incubated on ice for an additional 10 min. The mixture was then centrifuged at 20, 000 g and the supernatant removed. The DNA was then precipitated with the addition of 0.6 vol of isopropanol and recovered by centrifugation at 15, 000 g for 10 min. The DNA was washed with 70 % ethanol, spun down and placed in a vacuum to remove residual ethanol. The DNA was then dissolved in H_2O or TE (10 mM Tris, 1 mM EDTA). For the preparation of minipreps for analyzing DNA clones the same protocol was used, however all solutions were appropriately scaled down for 3 ml bacterial cultures.

For purification of plasmid DNA, CsCl/ethidium bromide equilibrium centrifugation was employed. The DNA pellet was resuspended in 4 ml of TE. To the DNA, 4.4 g of CsCl and 0.4 ml of a 10 mg/ml solution of ethidium bromide was added. The solution was centrifuged in an Optima MAX ultracentrifuge (Beckman Coulter) O/N at 100,000 rpm. The band representing the supercoiled plasmid DNA was removed with a needle and the ethidium bromide removed by mixing the DNA solution with 1 vol of isopropanol and removing the upper phase. This step was repeated 4-6 times until no ethidium bromide was visible. 3 vol of TE was then added to the DNA and the DNA precipitated with 0.6 vol of isopropanol. The DNA was then washed with 70 % ethanol and dissolved in an appropriate amount of H_2O.

Preparation of Competent *E. Coli* (DH5α) for DNA Electro-Transformation

A liter of LB (1 % Pepton (w/v), 0.5 % Bacto yeast extract (w/v), 0.5 % NaCl (w/v)) was inoculated with 10 ml of a fresh O/N culture of DH5α. Bacteria were grown at 37 °C with vigorous shaking to an OD_{600} of between 0.5-0.7. Bacteria were pelleted in sterile cold centrifuge bottles at 5,000 rpm for 10 min with an Avanti J-25I centrifuge (Beckman Coulter). Cells were then resuspended in 1 L of ice cold 10 % (v/v) glycerol and then spun down. This was repeated an additional 2 times using 0.5 L and 0.25 L of 10 % glycerol. The bacterial pellet was then resuspended in 3 to 4 ml of 10 % glycerol to a cell concentration of approximately 1-3 x 10^{10} cells/ml. The cells were frozen in aliquots on dry ice and stored at –80 °C.

For electroporation of bacteria, 40 µl of electro-competent DH5α cells were added to the DNA and mixed gently. The bacteria/DNA mixture was placed into a chilled 0.2 cm cuvette and the cuvette was then inserted into a *E. coli* pulser chamber. The cells were pulsed at 2.50 kV and immediately removed and placed into 1 ml of SOC solution (2 % Bacto pepton (w/v), 0.5 % Bacto yeast extract (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated for 1 h at 37 °C with shaking. A 300 µl aliquot of the transformed bacterial culture was plated onto solid 10 cm LB plates (1 % Bacto peptone (w/v), 0.5 % Bacto yeast extract (w/v), 0.5 % NaCl (w/v), 1.5 % agar (w/v)) containing the appropriate antibiotic and incubated O/N at 30 °C to allow colonies to form.

Tissue Culture

XR-V15B (Rathmell and Chu, 1994; Smider et al., 1994), HeLa and Cos7 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovin serum (FBS) at 37 °C in 5 % CO_2. Cells were maintained by splitting the cells every 3-5 days. For passaging, the cells were washed twice with phosphate-buffered saline (PBS) solution (137 mM KCl, 2.7 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) and 1 ml of trypsin was then added and the cells incubated at 37 °C for 2 min. DMEM supplemented with 10 % serum (5 ml) was then used to gently resuspend the cells. An aliquot of the cells representing 1/10 of the initial number of cells was added to a dish containing fresh medium.

Lipofectamine Transfection

Transfection with lipofectamine polycationic lipid transfection agent (Life Technologies) was performed essentially according to manufacturers protocols. Cells were seeded in 60 mm dishes and grown to 60-70 % confluence. Plasmids were diluted in 300 µl of Optimem (Life Technologies). To the DNA solution 6 µl of lipofectamine reagent was added and the sample vortexed briefly and incubated 1-2 h at room temperature (RT) to allow complexes to form. Cells were washed once with PBS and then with 2 ml of Optimem. The Optimem was then removed and 2 ml of fresh solution was added and the cells were incubated at 37 °C for 1-2 h. The Optimem was then removed and replaced

with 1.2 ml of fresh Optimem. The DNA/lipofectamine solution was then added to the dish and the cells were incubated for an additional 14 h at 37 °C. Following the incubation, the medium was replaced with DMEM supplemented with 10 % FBS and the cells incubated for 24 h to allow for protein expression.

Transient Expression of Recombinant Ku in XR-V15B and Cos7 Cells

XR-V15B or Cos7 cells were seeded on a 60 mm dish and allowed to reach 60 % confluence. Transfection was performed for 14 h in Optimem using lipofectamine with 100-500 ng of plasmid DNA. For nuclear import of nuclear localization signal deficient (NLS^-) Ku subunits, 100-500 ng of DNAs expressing the NLS^- subunit was co-transfected with a 2-fold excess of plasmid expressing the NL competent subunit.

Fluorescence and Indirect Immunofluorescence

To monitor enhanced green fluorescent protein (EGFP) fluorescence, following transfection, XR-V15B cells were incubated 6 hours in DMEM with serum, trypsinized and plated onto poly-L-lysine (500 μg/ml)-coated glass coverslips at 40 % confluence and incubated an additional 18 hours. Localization was assessed by fluorescence microscopy of living cells with a Zeiss Axioskop fluorescence microscope using a fluorescein isothiocyanate (FITC) filter. For indirect immunofluorescence, HeLa or transfected XR-V15B cells growing on poly-L-lysine-coated coverslips were fixed with 3 % paraformaldehyde (v/v) in PBS, pH 7.4 for 30 minutes at 4 °C, blocked 1 hour with 5 % (v/v) horse serum in PBS and incubated with the appropriate Ku antibody for 3-4

hours at RT. After washing five times with PBS, a FITC or Red-X conjugated secondary antibody was added for 1 hour at RT in the dark. Coverslips were washed, mounted and visualized under a fluorescent microscope using either an FITC or Texas Red filter. Data presented represents a minimum of 3 independent transfections and observation of at least 500 transfected cells.

Immunoprecipitation of Transiently Expressed Ku heterodimer

XR-V15B cells were cultured in 10 cm plates at 60-70 % confluence. Cells were transfected with equal amounts of DNAs (1 μg) of pBJ5Ku70 and either pEGFPKu80 or pEGFPKu80$_{A453H,V454H}$ for 14 hours in optimem with lipofectamine. Cells were then incubated an additional 24 hours in DMEM supplemented with 10 % FBS. Cells were washed with PBS and resuspended in 500 μl of immunoprecipitation (IP) buffer (50 mM HEPES pH 7.4, 10 % glycerol (v/v), 150mM NaCl, 1 % NP-40 (v/v), 1 mM EDTA, 0.5 mM dithiothreithol, 1 mM PMSF) supplemented with a cocktail of proteinase inhibitors (Roche Diagnostics). Cells were sonicated, cellular debris removed by centrifugation and an appropriate volume of cell extract was incubated O/N at 4 °C with mAb162 (200 μg/ml) at a 1:25 dilution. Preblocked protein G beads were added for 1 hour at 4 °C. The beads were washed 5 times with cold IP buffer and then boiled in SDS sample buffer prior to loading.

Fluorescence Recovery After Photobleaching (FRAP)

Measurement of nuclear export of proteins was performed by FRAP. Cos7 cells were transfected to express Ku proteins fused to the EGFP. Cells were treated in a Biotechs FCS2 environmental chamber maintained at 37 °C on a Biorad confocal microscope. EGFP signal in one nucleus of a multinucleated cell was irreversably bleached by focusing a laser on one nucleus and adjusting the power to maximum output. Fluorescence recovery was monitored over a period of 15 min. Prior to all experiments the cells were treated with cycloheximide (20 μg/ml final) for 1 h to prevent *de novo* protein synthesis. In some experiments cells were treated with leptomycine B (10 mM final) for 2 h prior to photobleaching.

Effect of MG132 and LMB Treatment on the Stability of Ku Subunits

Cos7 cells or XR-V15B cells transfected to express Ku70, Ku80 or both Ku70 and Ku80 were treated with leptomycine B (10 nM final), MG132 (2 μM final) or DMSO 24 h post transfection for 8, 8.5, 10.5 or 20 h as indicated. Whole cell extracts (100 μg of protein per lane) were subjected to Western analysis. Detection of expressed Ku70 and Ku80 in XR-V15B cells or endogenous Ku subunits in Cos7 cells to determine the amount of Ku in the extract was performed with Ku-specific antibodies N3H10 and 111, respectively. For XR-V15B cells, lamin B was used to control for protein loading.

Yeast Strains

Generation of stable yeast reporter strains YMpLacZNRE1, YMpLacZp53, YMpLacZOct and YMHisp53 was performed essentially as described in the MATCHMAKER One-Hybrid System User Manual (Clontech). All four strains are derived from the YM4271 strain (*MATa, ura3-52, his3-200, ade2-101, ade5, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4Δ, gal80Δ, ade5:::hisG*) (Clontech). For the YMpLacZNRE1 and YMpLacZOct strains four copies of the NRE1 sequence (upper strand: 5'-AACTGAGAAAGAGAAAGACGACA-3') and three copies of the octamer binding site (upper strand: 5'-ATGTAAAT-3') were cloned into the MCS of the pLacZi (Clontech) integration plasmid. 1μg of linearized plasmid was transformed into the YM4271 strain and yeast with the plasmid integrated into the genome by homologous recombination (at the *URA3* locus) were selected by *URA3* auxotophic selection. The YMpLacZp53 and YMHisp53 strains which contain three copies of the consensus p53 binding site as an upstream activating sequence (UAS) were constructed by integrating the p53Blue vector (Clontech) at the *URA3* locus and the p53HIS vector (Clontech) at the *HIS3* locus, respectively. Clones were verified by DNA sequencing and correct targeting of the reporter plasmids into the genome was confirmed by PCR. The Y190 reporter strain (*MATa, ura3-52, his3-Δ200,ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, URA3:::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ, cyh*^{r}*2, LYS2:::GAL1*$_{UAS}$*-HIS3*$_{TATA}$*-HIS3*) was originally obtained from Clontech.

Generation of the HDF2 Deficient Yeast Strain

The haploid HDF2 deficient strain (YMpLacZNRE1ΔHDF2) was generated by the gene disruption method with PCR generated targeting DNA. PCR primers were constructed with a 20 bp sequence complementary to regions upstream or downstream of the *HIS3* selectable gene marker of the pHisi-1 plasmid (Clontech) and 40 bp of homology upstream or downstream of the *HDF2* gene, forward primer 5'-CTAGATTACCGCATGTCCGTCAGGGCATTTGTTGTCATGCGAATTCCCGGGGAGCTCACG-3', reverse primer 5'-TTAGACCTTTTTTAATTATTGCTATTGTTTGGACTTCCCCGCATTAGGAAGCAGCCCAGT-3'. The PCR product was gel purified and approximately 1 μg of linear DNA was transformed into the YMpLacZNRE1 reporter strain. Stable transformants were selected on synthetic dropout (SD) minimal medium lacking histidine. Several colonies were harvested, grown in liquid culture and genomic DNA isolated. Deletion of *HDF2* was confirmed by PCR on genomic DNA with primers specific for sequences within the *HDF2* coding region, forward primer 5'-ATGTCAAGTGCGTCAACAACTTTCATCGTG-3', reverse primer 5'-AATGAGTCAGGAAGCTCTGGGATTTCAAAT-3'. Correct targeting of the *HIS3* selectable gene marker was also verified by PCR using one primer specific for the *HIS3* coding sequence and one primer specific for a DNA sequence immediately upstream of the *HDF2* coding region, forward primer 5'-

GAAATGATCGACTATGAGACCTTGAACCAG-3', reverse primer 5'-GGTGATGATCATTCTTGCCTCGCAGACAAT-3'.

Complementation of the TS and Growth Phenotypes of the HDF2 Deficient Yeast Strain

To determine whether human Ku could complement the TS phenotype of yeast lacking HDF2, YMpLacZNRE1 and YMpLacZNRE1ΔHDF2 yeast strains were transformed with plasmids expressing human Ku subunits or with empty parent plasmids. Yeast were grown at 30 °C on appropriate SD dropout medium for 5 days. A colony from each transformation was harvested and grown to equal density in liquid culture. The yeast suspensions were diluted to 10^{-2} and 10^{-5} with SD medium. Diluted yeast cultures (20 µl) were plated and the yeast incubated at 30 or 37 °C for 7 days. For the YMpLacZNRE1 and YMpLacZNRE1ΔHDF2 yeast transformed to express ADKu70/Ku80, a colony growing at 37 °C was picked and streaked onto a fresh plate and incubated further at 37 °C for 4 days.

For comparison of the growth rates between the YMpLacZNRE1 and YMpLacZNRE1ΔHDF2 strains, equal amounts of yeast in mid-log phase were diluted to 10^{-2} and 20 µl plated onto YPD medium and incubated for 3 days before assessing for growth. To examine the affect of human Ku on the growth rate of the YMpLacZNRE1ΔHDF2 strain, yeast were transformed to express ADKu70, ADKu70/Ku80 or with empty parent plasmids. Equal amounts of yeast were plated on appropriate SD medium and incubated at 30 °C for 4 days.

Yeast Transformation and Yeast-Two Hybrid Screening

For library screening, 20 large-scale transformations were performed with the Gal4DBDKu80$_{1-432}$ and Gal4DBDKu80$_{339-732}$ constructs at one time analyzing approximately 30,000 yeast colonies (135,000 library clones) for each construct. 100 μg of bait plasmid (pAS1-tetKu80$_{1-432}$ or pAS1-tetKu80$_{339-732}$) was mixed with 125 μg of prey plasmids and precipitated, washed with 70 % ethanol and resuspended in 100 μl of H_2O. For each transformation 5 μl of DNA was mixed with 5 mg of highly sheared salmon sperm DNA. 100 μl of competent yeast (Y190) was then added to the DNA and the contents gently mixed before adding 1 ml of PEG/LiAc/TE solution (40 % PEG$_{3350}$ (w/v), 100 mM LiAc, 10 mM Tris pH 8.0, 1 mM EDTA) and vortexing for 30 s. Tubes were then incubated at 42 °C for 10 min and then incubated in 5 ml of appropriate SD medium at 30 °C for 2-4 h with shaking. Yeast were spun down at 2,000 rpm with an Allegra 6R centrifuge (Beckman Coulter) for 3 min at RT and the medium was removed. Yeast from two transformations were plated on a single 20 cm plate of solid SD medium lacking leucine, tryptophan and histidine and containing a final concentration of 20 mM 3-amino-1,2,4-triazole (3-AT) to control for spurious *HIS3* reporter expression. The plates were then incubated at 30 °C for 8-10 days to allow colonies to form. A β-galactosidase filter assay was then performed on the yeast and colonies turning blue were harvested with a sterile toothpick and streaked onto a fresh plate and incubated for another 3 days before performing a second β-galactosidase filter assay. Yeast turning blue for a second time were harvested and grown in liquid culture at 30 °C and plasmid

DNA was isolated. The plasmid DNA recovered was then transformed into bacteria (DH5α) and colonies were selected on LB plates supplemented with 50 μg/ml (final) of ampicillin to select solely for bacteria containing the prey plasmids. Plasmid DNA was then isolated from the bacteria and transformed back into the original yeast strain, with either the original Gal4DBDKu80 bait plasmid or a nonspecific plasmid encoding for the Gal4DBD-Rab protein. Another β-galactosidase filter assay was performed. If colonies from both transformations resulted in blue colonies the clone was scored as a false positive, if colonies from neither transformation turned blue the clone was scored as a negative, and if only colonies transformed to express the original Gal4DBDKu80 bait turned blue the clone was scored as a positive.

Jurkat T-Cell cDNA Library

To identify putative Ku80 binding proteins using the yeast-two hybrid system a full-length Jurkat T-cell cDNA library was used for screening (Clontech). The library was estimated to contain 1 million independent clones. The cDNA clones were expressed as Gal4 activation domain fusions from the pGAD424 yeast expression plasmid (Clontech).

Calculation of 3-amino-1,2,4-triazole Factor

Since the Y190 strain used for the two-hybrid screening contained a *HIS3* reporter gene in addition to the *lacZ* reporter gene a positive clone would activate the *HIS3* reporter and stimulate colony growth in the presence of 3-amino-1,2,4-triazole (3-AT). To control for

spurious *HIS3* activation yeast were plated on minimal medium lacking histidine and containing 20 mM 3-AT. Therefore, in the presence of 3-AT the amount of colonies formed on the plates underestimates the actual library clones screened. To calculate the 3-AT factor, yeast were transformed with bait (containing the tryptophan selectable gene marker) and prey (containing the leucine selectable gene marker) plasmids and an equal amount of cells from the same transformation were plated on solid SD medium lacking leucine, tryptophan and histidine in the presence or absence of 3-AT (20 mM final). Yeast were incubated for 10 days at 30 °C and colonies formed on each plate were counted. To obtain the 3-AT factor, the number of colonies from the plate lacking 3-AT was divided by the number of colonies from the plate containing 3-AT. The 3-AT factor when multiplied by the amount of yeast colonies screened gives an estimation of the amount of library clones screened. The experiment was repeated 3 times and an average of the 3-AT factors was used.

β-galactosidase Filter Assay

Yeast colonies growing on solid medium were attached to a Whatman filter by gently placing a filter over the yeast and pressing the filter down with a plastic rod. The filter was then removed quickly with forceps and placed into liquid nitrogen for 1 min to permeabilize the cells. The filter was then removed and incubated at RT for 3 min before placing it over another filter (yeast colonies facing up) moistened with Z buffer (1.6 % Na_2HPO_4 $7H_2O$ (w/v), 0.55 % NaH_2PO_4 H_2O (w/v), 0.075 % KCl (w/v), 0.025 % $MgSO_4$ $7H_2O$ (w/v), pH 8.0) and containing 0.033 % 5-bromo-4chloro-3-indolyl-β-D-

galactopyranoside (w/v) (Clontech) and 0.27 % (v/v) of β-mercaptoethanol. The filters were then incubated at 30 °C and time required for colonies to turn blue was monitored.

Preparation of Competent Yeast

100 ml of sterile YPD medium (1 % Bacto yeast extract (w/v), 2 % Bacto peptone (w/v), 2 % dextrose (w/v)) supplemented with adenine (0.02 % final (v/v)) was inoculated with a yeast colony and incubated for 16-18 h at 30 °C with vigorous shaking. The yeast were then added to 1 L of YPD medium and incubated further until the OD_{595} reached between 0.6-0.8. Yeast were then pelleted at 2,000 rpm at RT, washed with 200 ml of H_2O and then resuspended in 50 ml of LISORB (100 mM LiAc, 10 mM Tris pH 8.0, 1 mM EDTA, 1 M Sorbitol) and incubated for an additional 20 min at 30 °C. Yeast were then centrifuged and the pellet resuspened in 2.5 ml of LISORB. DMSO was added (5 % (v/v) final) and aliquots were immediately frozen on dry ice and stored at –80 °C.

Preparation of Yeast Extracts for Western Analysis and Immunoprecipitation

A yeast colony was picked with a sterile toothpick and added to 5 ml of appropriate SD medium. The tube was vortexted for 5 s and the culture incubated O/N at 30 °C with shaking. The following day an aliquot of the yeast was diluted 50-fold in 25 ml of fresh medium and incubated at 30 °C until the OD_{595} reached between 0.6-0.8. The yeast were spun down at 2,000 rpm for 5 min and washed with 50 ml of H_2O. The yeast were pelleted and lysed by incubating at 95 °C for 5 min in the presence of 2XSDS sample

buffer. The extract was spun for 10 min at 13,000 rpm in a microcentrifuge (Heraeus Instruments) and the supernatant loaded on gel.

For preparation of yeast extracts for immunoprecipitation, yeast were resuspended in IP buffer and disintegrated by 3 bursts of vigorous shaking for 1 min in the presence of 1 volume of glass beads of 425-600 microns in size (Sigma) at 4 °C. The extracts were centrifuged at 13, 000 rpm and the supernatants removed and incubated with antibody 162 (0.008 μg/μl final).

Subcellular Fractionation

Subcellular fractionation was performed essentially as described (Andrews and Faller, 1991). HeLa or transfected XR-V15B cells cultured in a 10 cm dish were washed one time with PBS and then scraped into a microfuge tube containing cold PBS. Cells were pelleted and gently resuspended in 400 μl of cold buffer (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF). The cells were allowed to swell on ice for 10 minutes, and then vortexed for 10-15 seconds. Samples were centrifuged for 10-20 seconds, and the supernatant retained as the cytoplasmic fraction. Cellular lysis was monitored by trypan blue staining. To extract the nuclear proteins, the pellet was resuspended in 100-200 μl of cold extraction buffer (20 mM HEPES-KOH pH 7.9, 25 % glycerol (v/v), 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF), incubated on ice for 20 minutes and then centrifuged for 2-3 minutes at 4°C. The supernatant was retained as the nuclear fraction.

Co-Immunoprecipitation of Proteins with Endogenous Ku from HeLa Cell Extracts

HeLa cells were cultured to 80 % confluence, washed once with PBS and harvested. Cells were spun down and resuspended in IP buffer supplemented with a proteinase inhibitor cocktail (Roche Diagnostics) and then sonicated with two bursts of 10 s with a 2 min incubation on ice between bursts. The extracts were then spun down and the supernatants representing whole cell extracts were incubated with antibody 162 (0.008 μg/μl final) for 2-6 h at 4 °C. Protein A beads preblocked with bovine serum albumin (BSA) were then added for 1 h. Beads were then washed 5 times with IP buffer and incubated with 2XSDS sample buffer at 95 °C before loading the supernatant on gel. For co-immunoprecipitation of Sp1 with Ku, EDTA and the proteinase inhibitor cocktail was omitted from the IP buffer. For some Ku/TBP co-immunoprecipitation reactions ethidium bromide (200 μg/m final), highly sheared salmon sperm DNA (0.05 μg/μl or 0.45 μg/μl), DNaseI (3 units/μl) (GIBCO BRL) or NaCl (300 mM final) was added just prior to the addition of antibody. Immunoprecipitation of Ku from HeLa nuclear extracts was performed in the same way.

In Vitro Binding of Ku with TBP

For binding of Ku70 with TBP from HeLa WCEs, bacterially purified GST-Ku70 (300-500 ng) or GST-Ku70$_{\Delta 177\text{-}492}$ (500- 1000 ng) bound to glutathione sepharose beads (Amersham Pharmacia Biotech.) were incubated with 1 mg of HeLa WCE for 8 h at 4 °C with gentle shaking. Beads were then washed 5 times with binding buffer (12 % glycerol

(v/v), 15 mM HEPES pH 7.9, 60 mM KCl, 1.2 mM EDTA, 1.2 mM DTT, 0.12 mM PMSF, 0.1 % NP-40 (v/v)) and proteins were eluted from the beads with 2XSDS sample buffer and heating at 95 °C for 5 min. Beads were then spun down and the supernatants run on gel. For binding of purified 6xHisKu70 with GST-TBP, 100-300 ng of bacterially produced 6xHisKu70 was incubated with 500-800 ng of bacterially purified GST-TBP (Santa Cruz Biotech.) bound to beads and treated as above. The amount of GST-Ku70 proteins, 6xHisKu70 and GST-TBP was determined by comparing the intensities of the bands after SDS-PAGE and coomassie blue staining to a known amount of BSA.

Preparation of Recombinant GST-Ku70 and 6xHisKu70 Proteins

The pGEX-2T vector expressing full-length Ku70 or $Ku70_{\Delta 177-492}$ were transformed into BL21 bacteria and colonies selected on LB plates containing 50 μg/ml (final) of ampicillin. A colony was harvested and bacteria were grown in LB-ampicillin (50 μg/ml final) liquid culture at 37 °C to an OD_{600} of between 0.8-1.1. Expression of fusion proteins was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and incubating cultures for an additional 4 h at 37 °C. Bacteria were centrifuged at 5,000 rpm at 4 °C and the pellet resuspended in binding buffer. Bacteria were then sonicated with 3 bursts of 30 s with a 2 min incubation on ice between each burst. The samples were then centrifuged at 13,000 rpm for 10 min at 4 °C to eliminate bacterial debris and the supernatant was then incubated with glutathione Sepharose beads (Amersham Pharmacia Biotech.) or a Talon metal affinity column

(Clontech) at 4 °C for 1-1.5 h. The beads were washed 5 times with binding buffer and the purified proteins bound to the beads were stored at 4 °C.

SDS-PAGE Gels

Protein samples were dissolved in sample buffer (62.5 mM Tris-HCl pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 0.125 % (w/v) bromophenol blue) and denatured by boiling for 5 min at 95°C. Samples were loaded on SDS-PAGE gels which consisted of a stacking gel (4 % (w/v) 36.5:1 acrylamide:N,N'-methylenebisacrylamide, 62.5 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulfate, 0.1 % (v/v) TEMED) and a separating gel (8-12 % (w/v) 36.5:1 acrylamide:N,N'-methylenebisacrylamide, 375 mM Tris-HCl pH 8.8, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium persulfate, 0.05 % (v/v) TEMED). A mini-protein gel apparatus (Bio Rad) was used with a gel thickness of 0.75 mm. Gels were run at 100 volts for 60-120 min. Gels were analyzed by Western blotting or coomassie blue staining.

Western Blotting

Western blotting was performed as described (Burnette, 1981; Towbin et al., 1979). After separation by SDS-PAGE, proteins were electrophoretically transferred from the gel to a PVDF membrane (Immobilon P, Millipore Corp., Bedford, MA) in transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1 % (w/v) SDS, 20 % (v/v)

methanol). Prior to use the PVDF membrane was prepared by soaking in methanol for 10 s followed by transfer buffer for 2 min. Transfers were performed for 1-1.5 h with cooling at 100 V using a BioRad transblot apparatus. After transfer the membrane was rinsed 2 times in Tris-buffered saline (TBS)-Tween (TBS with 0.05 % (v/v) Tween-20) for 10 min at room temperature, then blocked with 10 % (w/v) skim milk in TBS-Tween for 1h at RT. The membrane was then incubated with a primary antibody O/N at 4°C in TBS-tween supplemented with 5 % skim milk. The primary antibodies were diluted between 1:400 to 1:1000 depending on the antibody. The membrane was then washed 3 times for 15 min with TBS-Tween at RT with gentle shaking. The membrane was then incubated for 1 h with either an horse radish peroxidase (HRP) conjugated anti-mouse or anti-rabbit secondary antibody diluted 1:50000 in TBS-Tween supplemented with 5 % skim milk. After washing as for the primary antibody, antibody-labeled proteins were detected by enhanced chemiluminescence using the Amersham ECL kit (Amersham Life Sciences Inc., Oakville, ON, Canada) as per the manufacturer's instructions. Stripping of antibodies from the membrane was performed by adding stripping solution (2 % (w/v) SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol) to the membrane and incubating for 30 min at 50 °C with gentle shaking. The membrane was then washed 3 times with TBS-Tween before treating as above.

Antibodies

Ku70 and Ku80 antibodies N3H10 and 111 (NeoMarkers), respectively, were used in immunofluorescence and immunoblot experiments. The Ku heterodimer-specific

antibody 162 (NeoMarkers) was used in immunofluorescence and immunoprecipitation experiments. GR was detected with antibody P-20 (Santa Cruz Biotech.) and the MEK-1 antibody (Santa Cruz Biotech.) was used as a nonspecific antibody for immunoprecipitation experiments. The remaining antibodies were used solely in Western blot analysis. In some instances Gal4 activation domain or Gal4 DNA binding domain Ku fusion proteins were detected with either an anti-Gal4 activation domain antibody (Clontech) or a HA-tag antibody (Y-11, Santa Cruz Biotech.). DNA-PKcs was detected with Ab-4 cocktail (NEOMARKERS). TAFIIp250, TAFIIp130 and TAFIp95/110 proteins were detected with antibodies 6B3 (Santa Cruz Biotech.), 4A6 (Santa Cruz Biotech.) and C-18 (Santa Cruz Biotech.), respectively. Sp1, TBP and lamin B were detected with antibodies 1C6 (Santa Cruz Biotech.), SI-1 (Santa Cruz Biotech) and M-20 (Santa Cruz Biotech.), respectively.

Part I: Introduction

Ku antigen binds avidly to many DNA structures including nicks, gaps, hairpins and virtually any double-to-single-stranded structural transition in DNA (Blier et al., 1993; Falzon et al., 1993; Mimori and Hardin, 1986; Paillard and Strauss, 1991). It is thought that for high affinity binding of Ku to DNA both Ku70 and Ku80 must be appropriately assembled into a heterodimer. However, under less stringent conditions the Ku70 monomer has been shown to bind to DNA *in vitro* (Allaway et al., 1990; Chou et al., 1992; Mimori and Hardin, 1986; Wang et al., 1998a). The C-terminus of Ku70 has been shown to have DNA binding activity in DNA immunoprecipitation assays and Southwestern experiments (Chou et al., 1992; Wang et al., 1998a). Also, the N-terminus of Ku70 when associated with Ku80 binds DNA in immunoprecipitation assays (Wang et al., 1998a). Further, it was reported that dimerization of Ku70 with Ku80 enhances the DNA binding activity of a Ku70 mutant lacking amino acids 543-609 from its C-terminus (Wang et al., 1998a).

In addition to Ku's high affinity binding to DNA structures, Ku has also been proposed to bind specifically to DNA sequences *in vitro* (Camara-Clayette et al., 1999; Genersch et al., 1995; Giampuzzi et al., 2000; Messier et al., 1993). However, the mechanism by which Ku interacts with many of these DNA sequences that are diverse in base composition is still unclear. We had previously demonstrated that Ku could bind directly with high affinity to a specific DNA sequence element found in the LTR of the MMTV (Giffin et al., 1996). We termed the sequence negative regulatory element 1 (NRE1) as we showed that this element is required for Ku/DNA-PKcs repression of

MMTV in response to steroid induced transcription in transient transfection assays (Giffin et al., 1999; Giffin et al., 1996). Despite many reports describing Ku binding to DNA, little is known about how Ku interacts with specific DNA binding sequences *in vivo* in a chromatin environment.

To study the Ku/NRE1 interaction and to identify the determinants of Ku required for binding to NRE1 *in vivo*, I chose to use the yeast one-hybrid system. This method was chosen as it has been successfully used to study the interaction of many proteins with their target DNA sequence. The system requires generation of a yeast reporter strain with a genomically integrated reporter gene with the target DNA sequence as the upstream activating sequence (UAS). The protein of interest is then expressed in the yeast fused to a strong activation domain (AD). For my experiments I chose to use the AD of the yeast Gal4 protein that strongly activates transcription of reporter genes in yeast when targeted to a yeast minimal promoter. Further, the Gal4 activation domain (Gal4AD) has been successfully used for other proteins in analyzing binding to their target DNA sequence.

I began by generating a yeast reporter strain containing four tandem copies of NRE1 as the UAS in the *E. coli lacZ* reporter gene. To detect Ku binding to NRE1 I expressed Ku fused to the Gal4AD. Binding of Ku to NRE1 would result in recruitment of the Gal4AD to the minimal promoter that in turn would result in activation of the *lacZ* gene. *LacZ* activation could be monitored by a β-galactosidase colormetric assay and activation was expected to directly reflect Ku binding to NRE1.

In the first part of my thesis I present the analysis of the unexpected finding that Ku expressed fused to the Gal4AD broadly activated reporter genes integrated into the yeast genome independent of a UAS (NRE1), yeast minimal promoter, reporter gene and

reporter integration site. In addition, in this section I also present data that show human Ku80 cannot substitute for the yeast homologue (HDF2) to rescue the temperature sensitive phenotype of a yeast strain deficient in HDF2. Further, I show that expression of the human Ku heterodimer was able to partially rescue the temperature sensitive phenotype but not the slow growth phenotype of HDF2 deficient yeast.

Part I: Results

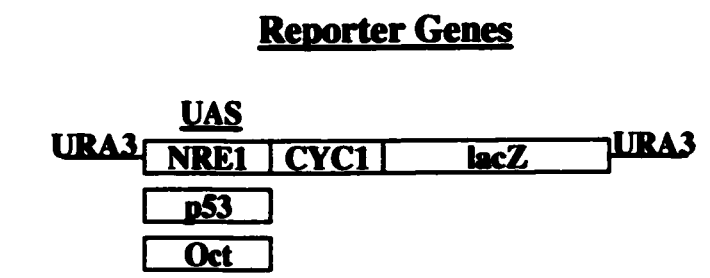
Human Ku70 with an ectopic activation domain activates *lacZ* transcription independent of a UAS

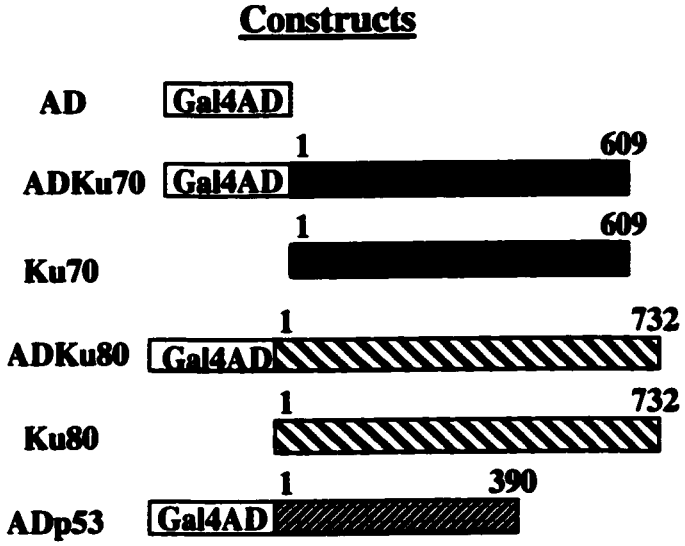
To begin an *in vivo* analysis of Ku binding to the MMTV NRE1 element, I expressed human Ku in a yeast strain containing the *E. Coli lacZ* reporter gene that has four tandem copies of NRE1 as an UAS (Fig. 3A, top). To ensure that Ku binding to the UAS would be detected through the activation of *lacZ* transcription, the AD of Gal4 was alternatively added to Ku70 and Ku80 (ADKu70 and ADKu80 Fig. 3A, bottom). *LacZ* reporter activation was measured using a β-galactosidase colony lift colormetric filter assay.

Co-expression of ADKu70 with Ku80 in cells containing the *lacZ* reporter with NRE1 as the UAS integrated at the *URA3* locus resulted in a strong activation of *lacZ* activity as seen by intense blue color in the β-galactosidase colony filter assay that was detected within 1-2 h of incubation (Fig. 3B). Ku-dependent activation also occurred when the Gal4AD was expressed fused to the N-terminus of Ku80 (Fig. 3B). However, in this instance *lacZ* activity was weaker, with the color becoming visible only after 18-24 h of incubation. *LacZ* activation was dependent on the Gal4AD domain as co-expression of Ku70 and Ku80 lacking the Gal4AD resulted in no induction of *lacZ* expression seen by lack of blue colonies even after a 24 h incubation with the colormetric reagent (Fig. 3B). The activation was however dependent on Ku as expression of the Gal4AD alone did not stimulate *lacZ* expression (Fig. 3B). Western analysis shows that

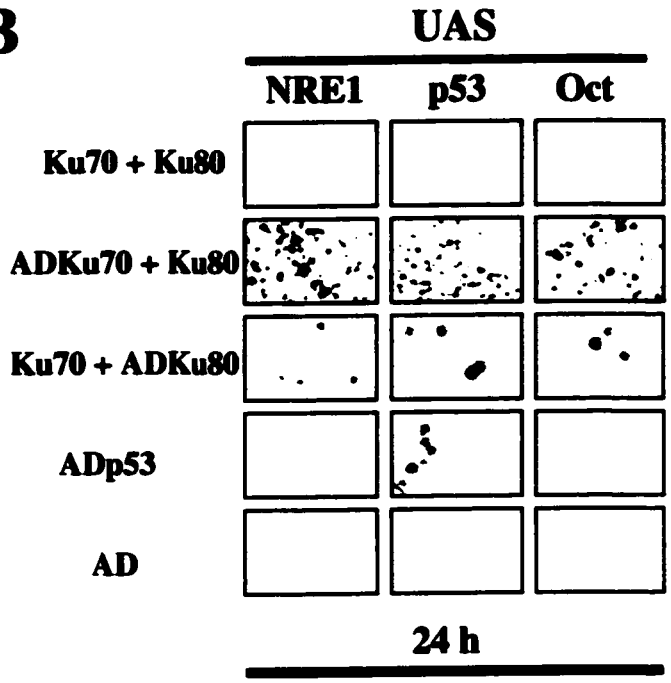
Figure 3. Human ADKu induces transcription of a *lacZ* reporter gene integrated into the yeast genome independent of a UAS. (A, top) Schematic illustration of reporter genes integrated into the yeast genome. Reporter genes were all targeted to the *URA3* locus of the YM4271 yeast strain and contained the *lacZ* gene driven by the *CYC1* minimal promoter flanked by either of three upstream activating sequences (UAS): NRE1, the p53 consensus binding site or an octamer binding site as indicated. (A, bottom) Schematic representation of the human Ku constructs expressed in the yeast reporter strains. Ku subunits were expressed as Gal4 activation domain (AD) fusion proteins or without the activation domain as shown. p53 was expressed as a Gal4AD fusion. (B) Transcriptional stimulation of the integrated reporters by yeast transformed to express Ku. Yeast reporter strains containing the NRE1, p53 or the octamer motif UAS (as indicated at the top) were transformed to express Ku, p53 or the Gal4 activation domain alone as indicated to the left. β-galactosidase filter assays were performed and blue colonies represent the activation of *lacZ*. Shown are pictures of filters following a 24 h incubation at 30 °C in the presence of X-gal. For expression of ADKu70/Ku80 and ADp53, blue colour was detected after 1-2 h of incubation, while for expression of ADKu80/Ku70 yeast turned blue after 18-24 h. (C) Western blot showing the relative expression levels of Ku subunits in yeast. Addition of the AD to the N-terminus of Ku70 does not effect the amount of ADKu70 or Ku80 present in yeast. Yeast whole cell extracts derived from the YMpLacZNRE1 strain transformed to express the indicated Ku constructs or extract of untransformed yeast (control) as indicated at the top were subjected to Western blot analysis. ADKu70 and Ku80 were detected with Ku70 and Ku80-specific antibodies N3H10 and 111, respectively.

A

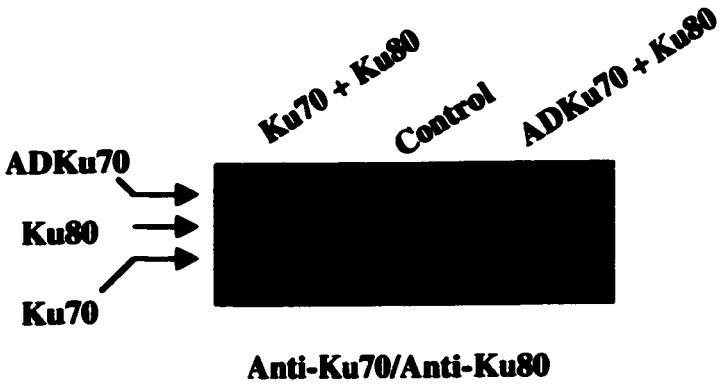

Reporter Genes
UAS
URA3
NRE1
CYC1
lacZ
URA3
p53
Oct


Constructs
AD
Gal4AD
1
609
ADKu70
Gal4AD
1
609
Ku70
1
732
ADKu80
Gal4AD
1
732
Ku80
1
390
ADp53
Gal4AD

B


UAS
NRE1
p53
Oct
Ku70 + Ku80
ADKu70 + Ku80
Ku70 + ADKu80
ADp53
AD
24 h

C

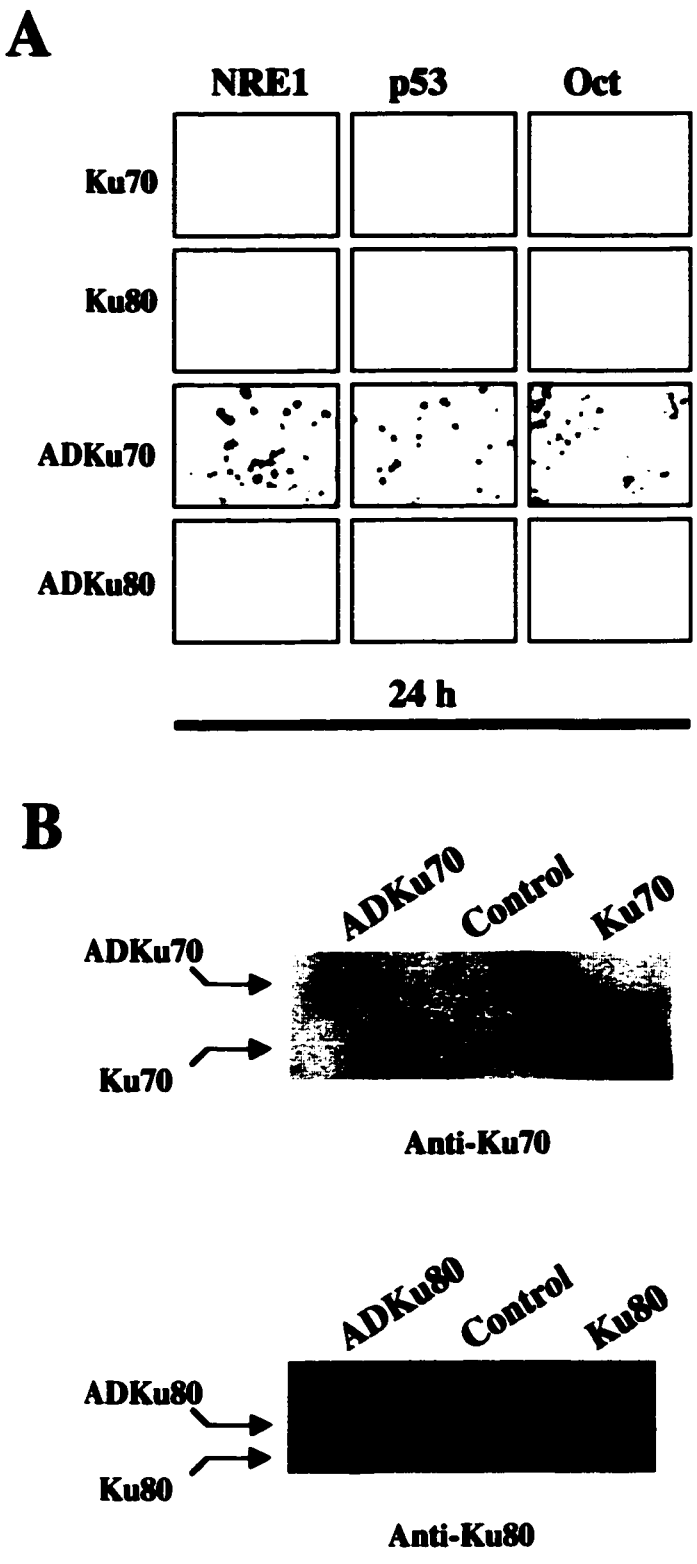

Ku70 + Ku80
Control
ADKu70 + Ku80
ADKu70
Ku80
Ku70
Anti-Ku70/Anti-Ku80

the addition of the AD to the Ku70 subunit had no significant effect on the protein levels of the Ku subunits in the yeast (Fig. 3C).

Unexpectedly, the *lacZ* activation was observed to be independent of NRE1, as ADKu induced *lacZ* activity to similar levels in two additional reporter strains containing oligonucleotides of the p53 consensus sequence and the octamer binding site as the UAS in the place of NRE1 (Fig. 3A, B). Neither the *lacZ* nor the additional sequences within the integrated sequences contained a sequence resembling the NRE1 element. Further, our lab has shown previously that Ku does not bind directly to an octamer motif *in vitro* (Giffin et al., 1996). This shows that the effects of Ku in this assay occurred independent of the NRE1 sequence element. The UAS-independent activation was specific for Ku as expression of the mouse p53 as a Gal4AD fusion (ADp53) only led to *lacZ* activation of the reporter gene containing the p53 consensus binding sequence as the UAS (Fig. 3B).

To ascertain whether this ectopic activation of *lacZ* expression required the Ku heterodimer I examined *lacZ* activity upon expression of ADKu70 and ADKu80 individually in the same yeast strains (Fig. 4A). Expression of Ku70 or Ku80 lacking the AD did not activate *lacZ* as seen by no blue colonies after a 24 h incubation (Fig. 4A). Expression of ADKu70 resulted in activation of *lacZ* seen by generation of blue colonies (Fig. 4A). However, no activation was observed when expressing ADKu80 alone (Fig. 4A). Moreover, addition of the AD did not significantly affect the levels of the individual Ku subunits in yeast as determined by Western analysis (Fig. 4B).

Figure 4. Human ADKu70 stimulates *lacZ* transcription when expressed in the absence of human Ku80. (A) Yeast strains containing the integrated reporters described in Figure 3 were transformed with plasmids expressing the individual Ku subunits alone or fused to the Gal4AD as indicated to the left. β-galactosidase filter assays were performed and blue colonies after a 24 h incubation represent *lacZ* activation. (B) Western blots showing expression levels of the Ku subunits expressed individually in yeast. An equal amount of whole cell extracts derived from the YMpLacZNRE1 strain transformed to express the indicated Ku subunit (at the top) was analyzed by Western blot. An extract from untransformed YMpLacZNRE1 was used as a negative control (control). Ku70 and Ku80 subunits were detected with antibodies N3H10 and 111, respectively.


A
NRE1
p53
Oct
Ku70
Ku80
ADKu70
ADKu80
24 h
B
ADKu70
Control
Ku70
ADKu70
Ku70
Anti-Ku70
ADKu80
Control
Ku80
ADKu80
Ku80
Anti-Ku80

To investigate the possibility that ADKu70-mediated induction of *lacZ* transcription was dependent on dimerization with the endogenous yeast Ku80 (HDF2) I repeated the experiments in a yeast strain containing the NRE1-*CYC1-lacZ* reporter gene in which the endogenous *HDF2* gene has been replaced by the *HIS3* selectable gene marker by homologous recombination (Fig. 5A). Deletion of the *HDF2* gene was confirmed by PCR using primers specific for internal regions of the *HDF2* coding sequence (Fig. 5B). Correct targeting of the *HIS3* marker was also determined by PCR with one primer specific for a region upstream of the *HDF2* coding sequence and one primer specific for the *HIS3* gene (Fig. 5B). Further, the HDF2 deficient strain displayed the characteristic temperature sensitive (TS) phenotype of HDF2 deficient yeast, with colonies forming at 30 °C but not at 37 °C (Fig. 5C).

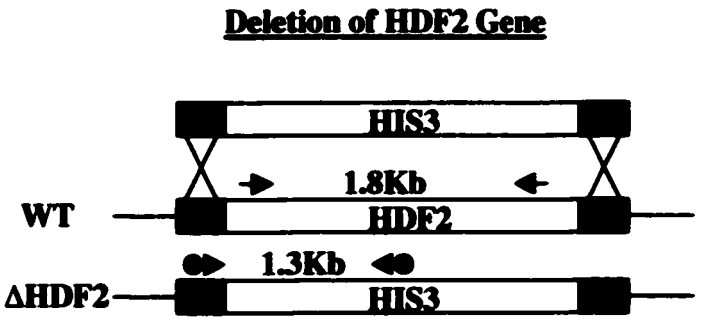
Deletion of *HDF2* had no effect on the ability of ADKu70 to activate *lacZ* as activation was observed with the HDF2 deficient strain (Fig. 6). These results demonstrated that the Ku70 monomer was sufficient for activation of the NRE1-*CYC1-lacZ* reporter. However, it was not a specific property of the ADKu70 monomer as the Ku70/ADKu80 heterodimer also activated *lacZ* (Fig. 3B).

ADKu70-dependent transcriptional activation is independent of minimal promoter, reporter gene or reporter gene integration site

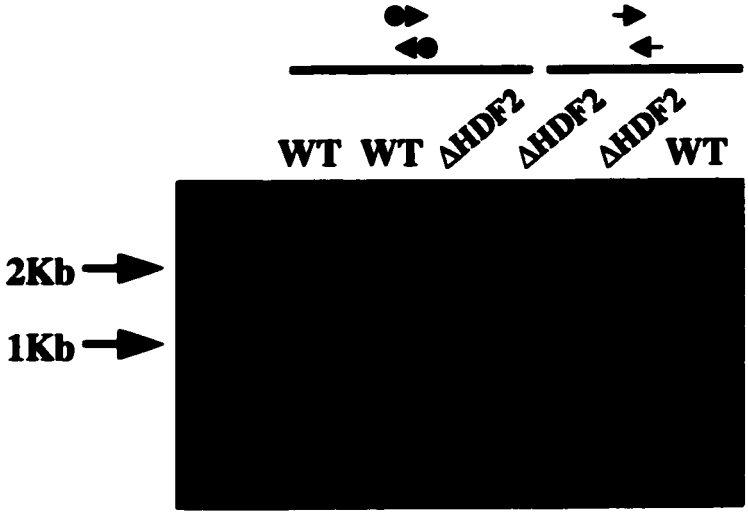
To determine if the activation was dependent on the *lacZ* gene, *CYC1* yeast minimal promoter or the *URA3* reporter integration site, I expressed ADKu70/Ku80 or ADKu70 in a yeast strain into which I integrated at the *HIS3* locus a *HIS3* reporter gene

Figure 5. Generation of the HDF2 deficient yeast reporter strain. (A) Deletion of the *HDF2* coding region was performed by the gene disruption method as schematically represented. 1 μg of linear DNA encoding for the *HIS3* selectable gene marker containing 40 base pairs of homology to regions upstream and downstream of the *HDF2* coding region was transformed into the YMpLacZNRE1 strain. Through a double homologous recombination event the *HDF2* gene was replaced by the *HIS3* selectable gene marker as shown. (B) Deletion of the *HDF2* gene was confirmed by PCR using genomic DNA from the parent (WT) and HDF2 deficient strain (ΔHDF2) with primers internal to the *HDF2* coding sequence as shown in A. A product of the correct size was only seen for the parent strain confirming deletion of the gene. PCR was also used to confirm correct targeting of the *HIS3* selectable gene marker with one primer specific for a sequence upstream of the *HDF2* coding region and one primer specific for the *HIS3* gene as depicted in A. For this primer set a PCR product of the expected sized was only generated with genomic DNA isolated from the HDF2 deficient strain (ΔHDF2). (C) The HDF2 deficient strain displays the characteristic temperature sensitive phenotype. The parent strain (WT) was able to form colonies at both 30 °C and 37 °C but the HDF2 deficient strain only forms colonies at 30 °C.

A


Deletion of HDF2 Gene
HIS3
1.8Kb
WT
HDF2
1.3Kb
ΔHDF2
HIS3

B


WT WT ΔHDF2 ΔHDF2 ΔHDF2 WT
2Kb
1Kb

C

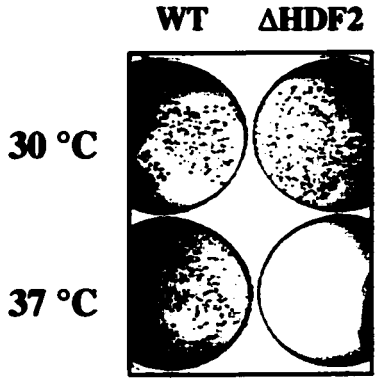
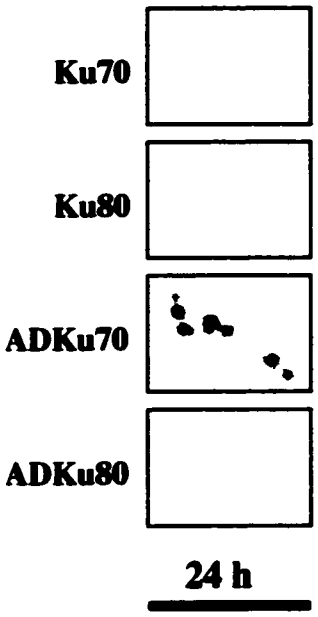

WT ΔHDF2
30 °C
37 °C

Figure 6. ADKu70 monomer activates *lacZ* in a yeast reporter strain deficient in HDF2. The YMpLacZNRE1 reporter strain deleted for the *HDF2* gene (NRE1ΔHDF2) was transformed to express the individual Ku subunits with or without the Gal4 activation domain as indicated to the left. β-galactosidase filter assays were performed to monitor the activation of the *lacZ* gene. Filters are shown after a 24 h incubation at 30 °C.

NRE1ΔHDF2



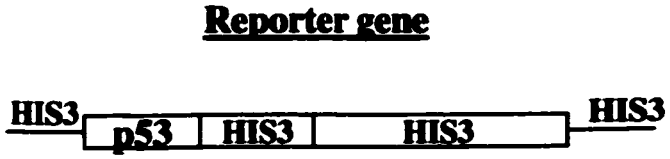
driven by the *HIS3* minimal promoter and p53 UAS as shown in Figure 7A. *HIS3* reporter expression was assessed by recording the growth of yeast on medium lacking histidine and containing increasing concentrations of the yeast His3 protein competitive inhibitor, 3-amino-1,2,4-triazol (3-AT). The yeast strain was transformed to express ADKu70/Ku80, ADKu70, ADp53 or the Gal4AD alone.

Yeast transformed with each of the constructs grew equally well on medium containing histidine and lacking 3-AT (Fig. 7B). In the absence of histidine and the presence of 10 mM 3-AT, yeast expressing the Gal4AD was markedly impaired for growth as expected in the absence of induction of the *HIS3* reporter (Fig. 7B). Yeast expressing ADKu70/Ku80 or ADKu70 alone resulted in *HIS3* activation reflected by growth of the yeast at 20 and moderate growth at 50 mM 3-AT (Fig. 7B). These results demonstrated that the Ku-dependent activation was not specific for a minimal promoter, reporter gene or particular reporter integration site.

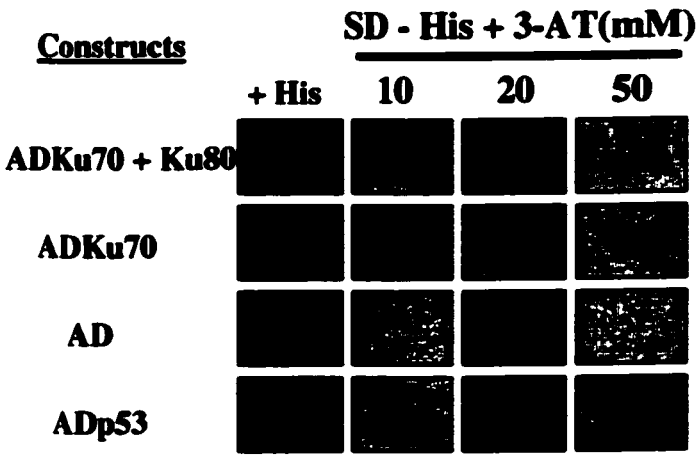
By comparison to ADKu70/Ku80 the p53-*HIS3*-*HIS3* reporter containing the p53 binding sequence as the UAS was activated more strongly by ADp53 as yeast expressing ADp53 grew normally even at 50 mM 3-AT (Fig. 7B). However, I observed no significant difference in levels of reporter activation between ADp53 and ADKu70/Ku80 with the p53-*CYC1*-*lacZ* reporter (Fig. 3B). These results suggest that the p53-*CYC1*-*lacZ* reporter is more strongly activated by ADKu70/Ku80 relative to the p53-*HIS3*-*HIS3* reporter.

Figure 7. ADKu70/Ku80 and ADKu70 stimulate transcription of a *HIS3* reporter gene. (A) Schematic illustration of the *HIS3* reporter gene used to measure ADKu-dependent transcriptional activation. The *HIS3* reporter containing the *HIS3* minimal promoter and p53 binding sequence as the upstream activating sequence was integrated at the *HIS3* locus of the YM4271 yeast strain. (B) ADKu70/Ku80, ADKu70 and ADp53 activate the p53-*HIS3*-*HIS3* reporter. An equal amount of recombinant *HIS3* reporter strain transformed to express the various constructs indicated to the left was streaked onto solid synthetic dropout (SD) medium either containing (+His) or lacking histidine (-His) and containing increasing concentrations (10, 20 or 50 mM) of the yeast His3 protein competitive inhibitor, 3-amino-1,2,4-triazole (3-AT). Yeast growth was assessed after a 6-day incubation period at 30 °C in the dark.

A


Reporter gene
HIS3
p53
HIS3
HIS3
HIS3

B


Constructs
SD - His + 3-AT(mM)
+ His
10
20
50
ADKu70 + Ku80
ADKu70
AD
ADp53

To further explore the pleiotrophic Ku-dependent transcriptional activation I tested the ability of ADKu70/Ku80 and ADKu70 to activate transcription in another yeast strain (Y190) with a *lacZ* reporter transcribed by the *GAL1* minimal promoter containing the Gal1 binding site as the UAS and integrated at the *URA3* locus. I observed that expression of ADKu70/Ku80 or ADKu70 alone also activated *lacZ* in this strain (Table I). This data demonstrated a general interaction of human Ku with genes having the potential to be transcribed in yeast.

A summary of the activation of reporter genes integrated in the genome of various yeast strains by key Ku constructs is presented in Table 1. The table summarizes the data of β-galactosidase assays and 3-AT titration experiments used to determine *lacZ* and *HIS3* reporter gene activation, respectively.

Ku-dependent activation of yeast genes is a specific property of human Ku70 that is not conserved in the yeast homologue

To investigate whether yeast Ku might have a generalized ability to interact broadly with genes that was similar to my observation with human Ku, I expressed the yeast Ku70 homologue, HDF1, in reporter strains as a Gal4AD fusion protein in the same configuration I expressed human Ku70. No reporter activation was observed when expressing ADHDF1 in the HDF2 deficient strain containing the NRE1-*CYC1-lacZ* reporter or in the parent strain that expresses endogenous HDF2 and where the yeast ADHDF1/HDF2 heterodimer was expected to form normally (Fig. 8A). ADHDF1 also failed to activate transcription of the p53-*HIS3-HIS3* and Gal1-*GAL1-lacZ* reporter genes

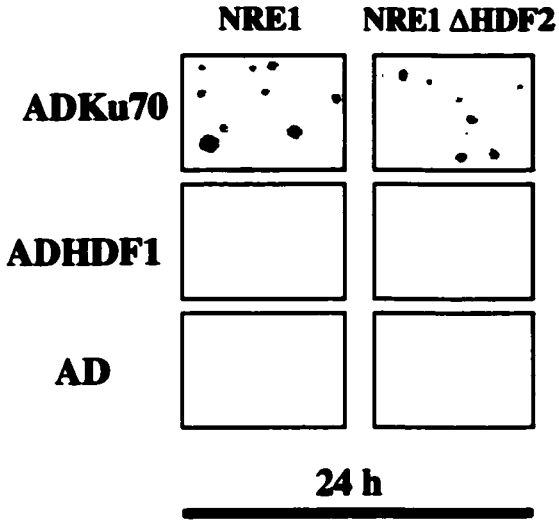
Table 1 Summary of Ku-dependent transcriptional activation of reporter genes in yeast. Shown are the results of β-galactosidase filter assays and 3-amino-1,2,4-triazole titrations used to determine activation by Ku of the *lacZ* and *HIS3* reporter genes integrated into the yeast genome, respectively. The upstream activating sequences (UAS) and yeast minimal promoters for the *lacZ* and *HIS3* reporter genes of each yeast strain is indicated. Yeast were transformed to express the constructs indicated to the left. Activation (+) of the reporter genes represents an induction in expression above that of the untransformed yeast. No activation (-) of the reporter gene represents no stimulation of reporter expression when compared to the untransformed yeast. N/D- not determined.

Table 1

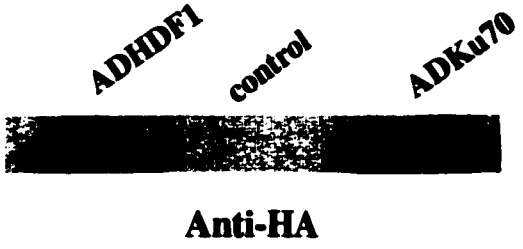
Expressed Constructs	Yeast Strain: Minimal Promoter/Reporter Gene				
	YMpLacZ CYC1/lacZ			Y190 GAL1/lacZ	YMHisp53 HIS3/HIS3
UAS:	NRE1	p53	Oct	Gal1	p53
Ku70 + Ku80	-	-	-	-	-
ADKu70 + Ku80	+	+	+	+	+
Ku70 + ADKu80	+	+	+	N/D	N/D
Ku70	-	-	-	-	-
Ku80	-	-	-	-	-
ADKu70	+	+	+	+	+
ADKu80	-	-	-	-	-
$ADK70_{58-609}$	-	-	-	-	-
$ADK70_{58-609}$ + Ku80	-	-	-	-	-
$ADK70_{1-491}$	-	-	-	-	-
$ADK70_{1-491}$ + Ku80	+	+	+	+	+
$ADK70_{\Delta 177-492}$	+	+	+	+	+
ADHDF1	-	-	-	-	-
Gal4AD	-	-	-	-	-

Figure 8. ADHDF1 does not activate *lacZ*. (A) The YMpLacZNRE1 strain (NRE1) and the YMpLacZNRE1ΔHDF2 strain (NRE1ΔHDF2) deleted for the *HDF2* gene were transformed to express ADKu70, ADHDF1 or the Gal4 activation domain (AD) as indicated to the left. Yeast transformants were subjected to a β-galactosidase assay to determine transcriptional induction of *lacZ*. Filters are shown after a 24 h incubation at 30 °C for both strains and blue colonies represent *lacZ* activation. (B) Western blot showing similar expression levels of ADHDF1 and ADKu70 in yeast. Equal amounts of whole cell extracts from the YMpLacZNRE1ΔHDF2 yeast strain transformed to express ADHDF1 or ADKu70 as indicated at the top were subjected to Western blot analysis. Untransformed yeast extract was included as a negative control (control). Ku70 proteins were detected with a HA-tag antibody (Y-11).

A


NRE1
NRE1 ΔHDF2
ADKu70
ADHDF1
AD
24 h

B


ADHDF1
control
ADKu70
Anti-HA

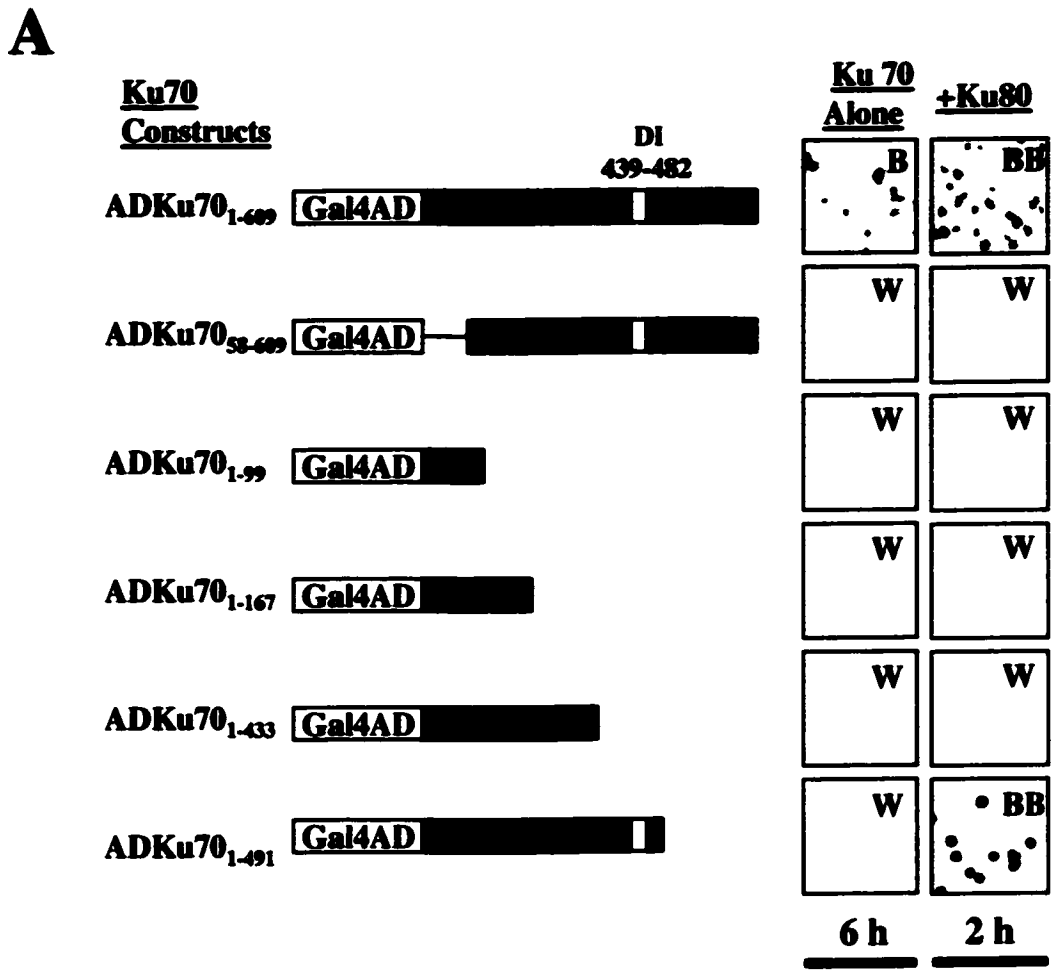
(Table I). Western analysis showed similar levels of expression of the human ADKu70 and ADHDF1 excluding protein expression levels as a possible explanation for the absence of activation (Fig. 8B). These results strongly suggest that the Ku-dependent activation was a property of human ADKu70 that was not conserved in the yeast homologue.

The components of Ku required for activation correlate with the portions needed for DNA binding

To delimit the requirements within Ku70 that were required for transmission of the effects of the Gal4AD on yeast genes examined in this study, I generated a series of N and C-terminal deletion mutants of ADKu70. I expressed these constructs in the HDF2 deficient strain alone or in combination with human Ku80 and measured their ability to activate the NRE1-*CYC1-lacZ* reporter gene with my colormetric assay (Fig. 9A).

Expression of full-length ADKu70 alone resulted in blue colonies after 4-6 h of incubation. However, co-expression of ADKu70 and Ku80 enhanced the induction of *lacZ* leading to the visualization of blue colonies in 1-2 h (Fig. 9A). The increase in *lacZ* activation directly correlated with the level of ADKu70 in yeast. Co-expression of ADKu70 with Ku80 resulted in a 3 to 4-fold increase in the level of ADKu70 that accumulated in the cell (Fig. 9B, top). This is in agreement with previous reports indicating that Ku70 and Ku80 dimerization dramatically stabilizes Ku70 and Ku80 monomers in mammalian cells (Gu et al., 1997a).

Figure 9. The N-terminus of ADKu70 is required but not sufficient for activating *lacZ*. (A) Shown to the left are the N and C-terminal deletion constructs that were tested for their potential to activate *lacZ* transcription in the YMpLacZNRE1ΔHDF2 reporter strain. Some constructs contain the previously identified interface of Ku70 required for dimerization with Ku80 (DI). Ku70 constructs were expressed alone or in combination with Ku80 as shown and *lacZ* induction was assessed by β-galactosidase filter assay. Pictures of filters were taken after 2 or 6 h of incubation as shown to the right. *LacZ* activation was scored as follows: BB, colonies turned blue between 1-2 h; B, colonies turned blue in 4-6 h; W, no blue colonies were observed after 24 h. (B) Western blots of whole cell extracts derived from the YMpLacZNRE1ΔHDF2 strain transformed to express the constructs indicated at the top showing relative levels of ADKu70 proteins. Extracts from untransformed YMpLacZNRE1ΔHDF2 were included as negative controls (control). ADKu70 constructs were detected with either an anti-Gal4 activation domain antibody (top) or the Ku70-specific antibody, N3H10 (bottom).


A
Ku70 Constructs
DI 439-482
Ku 70 Alone
+Ku80
ADKu70 1-609
Gal4AD
B
BB
ADKu70 58-609
W
ADKu70 1-99
ADKu70 1-167
ADKu70 1-433
ADKu70 1-491
• BB
6 h
2 h


B
ADKu70
ADKu70+Ku80
Control
ADKu70 1-491
ADKu70 1-491+Ku80
Anti-Gal4AD
Control
ADKu70 58-609
ADKu70
Anti-Ku70

A short deletion of 57 amino acids from the N-terminus of ADKu70 completely abolished *lacZ* activation (Fig. 9A). Since the N-terminal deletion mutant is expressed at only marginally lower levels than the full-length ADKu70 the lower expression cannot completely account for the lack of activation (Fig. 9B, bottom). Furthermore, the activation was not restored by co-expression of Ku80 even though this significantly increased the level of $ADKu70_{58-609}$ present in the cell through dimerization with Ku80 (Fig. 9A, data not shown). Dimerization was confirmed by co-immunoprecipitation (shown in Fig. 13B). Moreover, I determined that the N-terminus of ADKu70 was not sufficient for the induction of *lacZ* by ADKu70 as $ADKu70_{1-99}$, $ADKu70_{1-167}$ and $ADKu70_{1-433}$ each failed to activate *lacZ* when expressed alone or in combination with Ku80 (Fig. 9A). $ADKu70_{1-491}$ also did not activate *lacZ* when expressed alone despite being expressed to higher levels than ADKu70 (Fig. 9B, top), however co-expression of this construct with Ku80 resulted in blue colonies in 1-2 h consistent with $ADKu70_{1-491}$ expression levels (Fig. 9B, top). The restoration of *lacZ* activation of $ADKu70_{1-491}$ is due to its ability to dimerize with Ku80 as this larger N-terminal fragment contains amino acids 439-482 which represent the major region of Ku70 that mediates dimerization with Ku80 and this sequence was deleted in the shorter ADKu70 N-terminal fragments. Formation of the $ADKu70_{1-491}$ /Ku80 heterodimer was also confirmed by co-immunoprecipitation (data not shown). Thus it appeared that determinants within Ku80 substituted for the C-terminus of Ku70 in promoting the activation of *lacZ* by ADKu70.

As deletion of both the N and C-terminus of the ADKu70 abrogated its ability to activate transcription I generated ADKu70 internal deletion mutants to determine if the middle portion of the Ku70 monomer was also required to induce *lacZ* transcription.

Deletion mutants ADKu70$_{\Delta 229\text{-}362}$, ADKu70$_{\Delta 177\text{-}362}$, and ADKu70$_{\Delta 177\text{-}492}$ expressed alone all activated transcription of *lacZ* with colonies turning blue in 1-2 h which was comparable to the ADKu70/Ku80 heterodimer and stronger than the full-length ADKu70 monomer (Fig. 10A). Comparing the protein levels in yeast of ADKu70 to ADKu70$_{\Delta 177\text{-}492}$ revealed that the internal deletion mutant was expressed at a higher level and approached the level of ADKu70 when expressed with Ku80 (Fig. 10B). Thus, at least in yeast, deletion of the middle portion of ADKu70 appears to significantly stabilize the monomer. Moreover, the level of activation by the internal deletion mutants was not affected further by co-expression of Ku80 (Fig. 10A). A larger internal deletion mutant ADKu70$_{\Delta 177\text{-}584}$ failed to activate *lacZ* regardless of the presence of Ku80, revealing that determinants between amino acids 492 to 584 were essential for activation by ADKu70 (Fig. 10A).

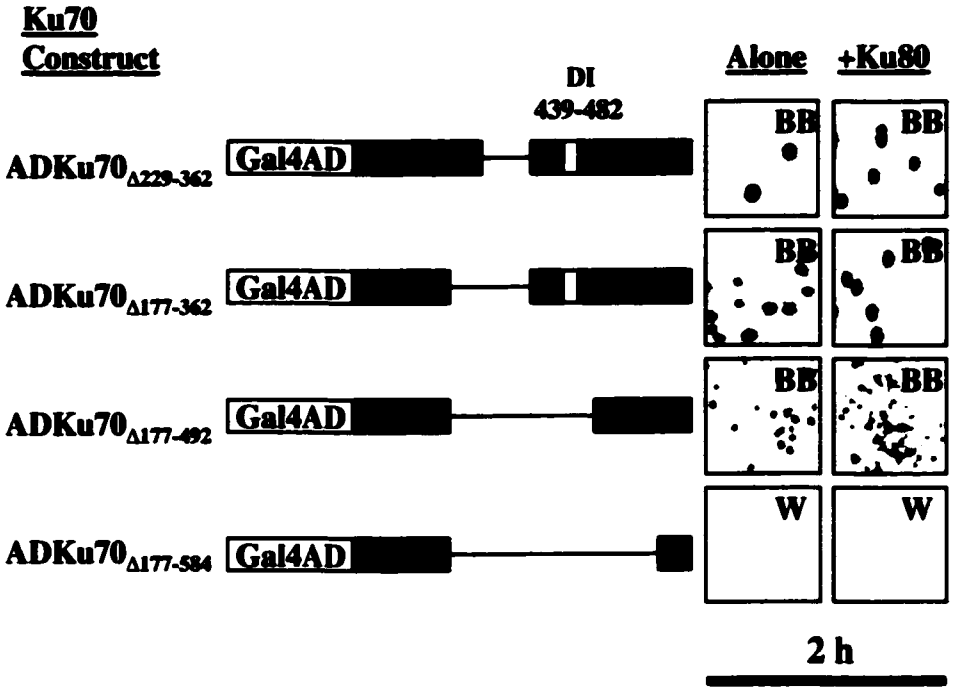
All ADKu mutants that activated the NRE1-*CYC1-lacZ* reporter also activated the p53-*HIS3-HIS3* and Gal1-*GAL1-lacZ* reporter genes (Table I). Together my results show that the ADKu70 monomer has the ability to broadly activate reporter genes integrated into the yeast genome and the activation observed appears to be dependent on a common feature shared by the *CYC1*, *HIS3* and *GAL1* yeast minimal promoters.

Ku70$_{58\text{-}609}$ mutant that does not pleiotrophically activate reporter genes in yeast is associated less tightly with the nucleus upon biochemical fractionation

I had previously shown that expression of the Ku70 monomer in rodent XR-V15B cells that lack Ku80 remained predominately in the nuclear fraction upon biochemical

Figure 10. Portions of ADKu70 required for *lacZ* activation correlate with regions needed to bind DNA. (A) ADKu70 internal deletion mutants were expressed in the YMpLacZNRE1ΔHDF2 strain alone or in combination with Ku80 and *lacZ* transcriptional stimulation was determined by β-galactosidase filter assay. A schematic illustration of the ADKu70 constructs expressed is presented to the left. Shown to the right are filters of β-galactosidase assays of yeast transformed with the corresponding ADKu70 mutants either alone or with Ku80 as indicated. Filters are shown after a 2 h incubation. *LacZ* activation was scored as follows: BB, colonies turned blue after 1-2 h; W, no blue colonies were observed after 24 h. (B) Western blot showing the expression levels of ADKu70 and $ADKu70_{\Delta 177-492}$ in yeast. An equal amount of whole cell extract from the YMpLacZNRE1ΔHDF2 strain transformed to express ADKu70, $ADKu70_{\Delta 177-492}$ or untransformed yeast (control) was subjected to immunoblot analysis to compare levels of Ku70 proteins. ADKu70 proteins were detected with a HA-tag antibody (Y-11).

A


Ku70
Construct
Alone
+Ku80
DI
439-482
ADKu70Δ229-362
Gal4AD
BB
BB
ADKu70Δ177-362
Gal4AD
BB
BB
ADKu70Δ177-492
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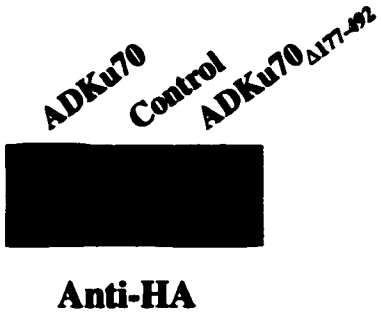
BB
BB
ADKu70Δ177-584
Gal4AD
W
W
2 h

B

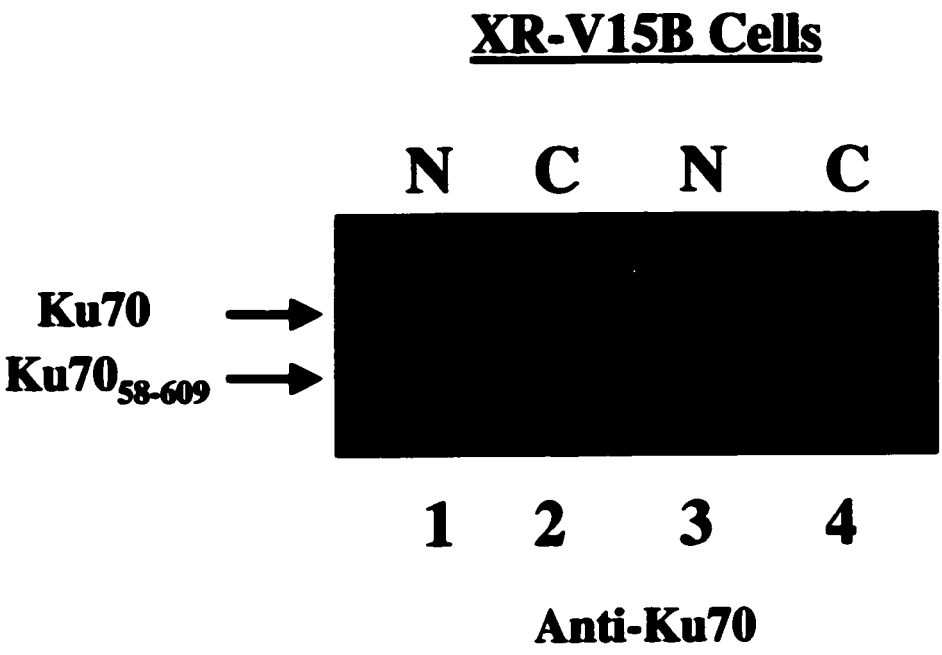

ADKu70
Control
ADKu70Δ177-492
Anti-HA

fractionation of the cells into nuclear and cytoplasmic fractions (see Fig. 16B). Since fractionation of cells by hypotonic lysis results in the release from the nucleus of many freely circulating or weakly bound proteins this result indicated that a large proportion of Ku70 is tightly associated with the nucleus in mammalian cells. I therefore wished to test if a mutation in Ku70 that disrupted its ability to broadly activate reporter genes in yeast would affect the degree in which Ku70 is retained in the nucleus upon biochemical fractionation. The full-length Ku70 monomer and a deletion mutant lacking amino acids 1-57 from its N-terminus were transiently expressed in XR-V15B cells. Ku70 was predominately found in the nuclear fraction (Fig. 11, compare lanes 1 and 2) whereas $Ku70_{58-609}$ was equally distributed between the nuclear and cytoplasmic fractions (Fig. 11, compare lanes 3 and 4) demonstrating that $Ku70_{58-609}$ is less tightly associated with the nucleus than the WT Ku70. This result indicated that the extreme N-terminus of Ku70 that was required for ADKu70 to globally activate reporter genes in yeast was also necessary to bind a significant proportion of the Ku70 monomer to the nucleus in mammalian cells.

Expression of human Ku heterodimer but not the Ku80 monomer partially rescues the temperature sensitive phenotype of a HDF2 deficient yeast strain

Yeast that have been genetically altered to lack Ku70 and/or Ku80 in *S. cerevisiae* show a reduced growth rate when grown at 30 °C compared to the parent strain that expresses Ku. In addition, these cells display a temperature sensitive (TS) phenotype as they die when placed at 37 °C. For HDF1 deficient yeast it was reported that these yeast

Figure 11. The extreme N-terminus of Ku70 is required for tight association of the Ku70 monomer with the nucleus. Distribution of Ku70 constructs upon biochemical fractionation of mammalian cells into nuclear and cytoplasmic fractions. Western blot of equal nuclear (N) and cytoplasmic (C) fractions of XR-V15B cells transiently transfected to express Ku70 or $Ku70_{58-609}$. Ku70 proteins present in the nuclear fractions are shown in lanes 1 and 3 and those found in the cytoplasmic fractions are represented in lanes 2 and 4. Ku70 proteins were detected with antibody N3H10.


XR-V15B Cells
N C N C
Ku70
Ku70 58-609
1 2 3 4
Anti-Ku70

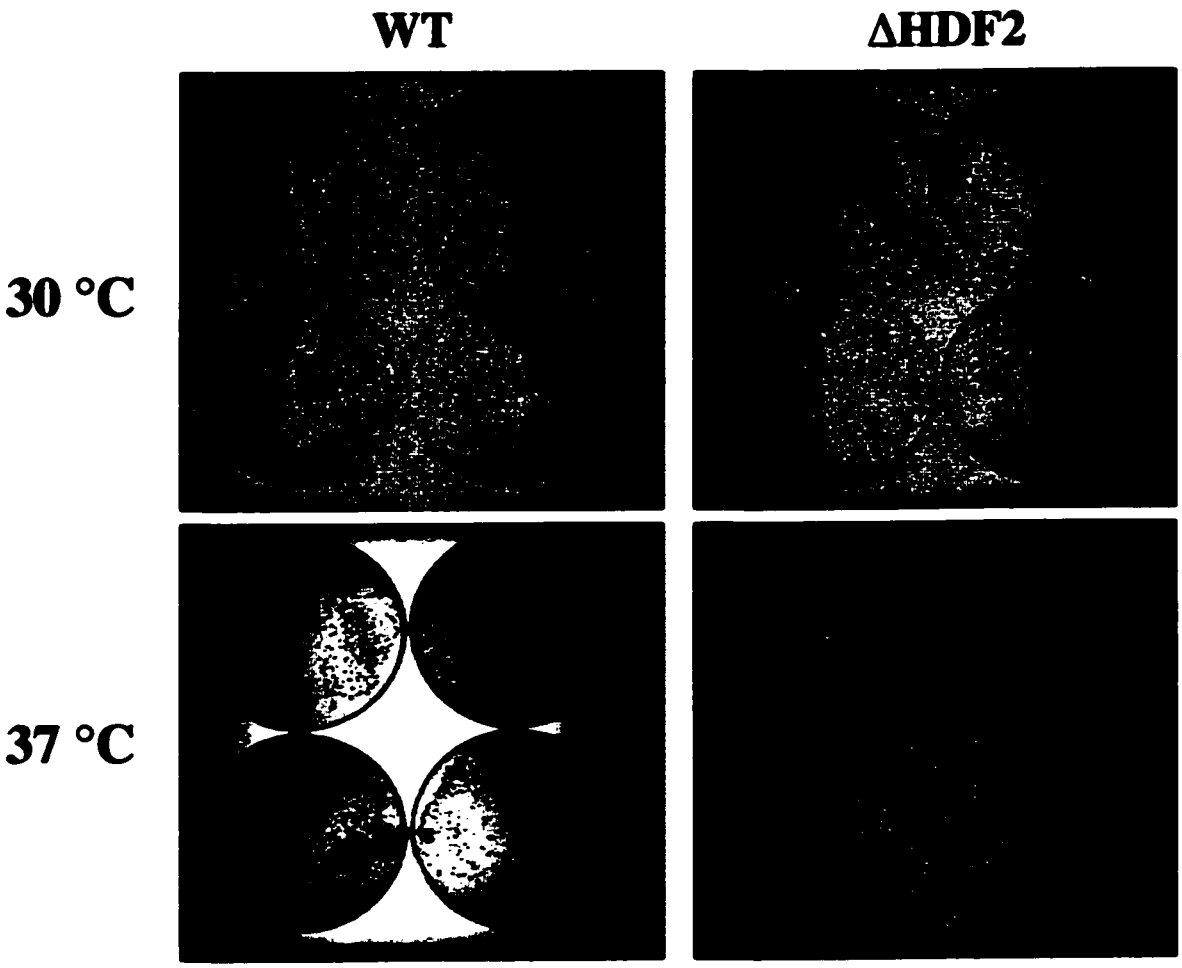
do form colonies at the restrictive temperature but fail to continue to propagate when the yeast colonies grown at 37 °C are streaked onto a fresh plate and incubated further at the restrictive temperature (Barnes and Rio, 1997). Ectopic expression of human Ku70 in HDF1 deficient yeast was demonstrated to rescue the TS phenotype of these yeast suggesting that the human Ku70 can substitute for the HDF1 to rescue the temperature sensitivity (Barnes and Rio, 1997). To date, the mechanism by which Ku deficiency translates to the slow growth and TS phenotypes is not known.

To further understand the nature of the TS phenotype in Ku deficient yeast I wanted to determine whether human Ku80 can substitute for HDF2 to rescue the TS phenotype in the YMpLacZNRE1ΔHDF2 strain in which I deleted the *HDF2* gene (see Fig. 5). I transformed the HDF2 deficient (ΔHDF2) and parent (WT) strain to express human ADKu70, Ku80, ADKu70 and Ku80 or the empty parent plasmids. Equal amounts of the transformed strains were assessed for growth over 7 days.

The WT strain grew at both temperatures for all transformations (Fig. 12A, left panels). The HDF2 deficient strain also formed colonies at 30 °C for all transformations (Fig. 12A, top right panel). Expression of Ku80 in the yeast lacking HDF2 did not rescue the TS phenotype, as colonies were not formed at 37 °C (Fig. 12A, bottom right panel). Colonies also failed to form in this strain when transformed to express ADKu70 or the empty parent plasmids (Fig. 12A, bottom right panel). In contrast to previous reports with a HDF1 deficient strain where yeast initially grew at 37 °C but failed to continue to propagate once restreaked (Barnes and Rio, 1997), I failed to observe any colony formation with the HDF2 deficient yeast (Fig. 12, bottom right panel). Yeast colonies

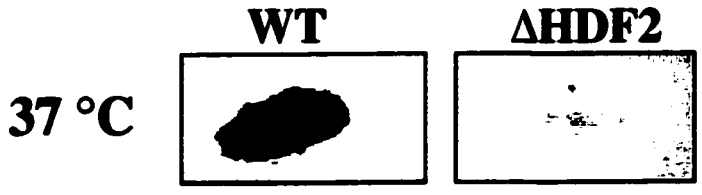
Figure 12. Effects of human Ku on growth of HDF2 deficient yeast. (A) The YMpLacZNRE1ΔHDF2 strain deficient in HDF2 (ΔHDF2) or the parent strain (WT) was transformed to express the indicated Ku constructs or with parent plasmids and grown on appropriate solid synthetic dropout medium at 30 °C for 5 days. A colony from each transformation was harvested and grown at 30 °C in liquid culture to mid-log phase before plating two dilutions on solid medium. Plates were incubated at 30 or 37 °C for 7 days. Yeast were transformed as follows: A, parent vectors; B, Ku80; C, ADKu70; D, ADKu70 +Ku80. (B) A yeast colony from the WT and ΔHDF2 strains transformed to express ADKu70 + Ku80 growing at 37 °C shown in panel A were streaked onto a fresh plate and incubated at 37 °C for an additional 4 days.

A



7-day incubation

B



4-day incubation

were also not observed when plating the HDF2 deficient strain in the absence of any transformation (see Fig. 5, C).

Surprisingly, yeast transformed to express both ADKu70 and Ku80 did form visible colonies at the restrictive temperature, albeit smaller than the WT strain (Fig. 12A, bottom right panel). However, upon streaking the ADKu70/Ku80 expressing ΔHDF2- yeast colonies to fresh medium and incubating further at 37 °C the yeast fail to continue propagating (Fig. 12B) indicating that the human Ku can only partially rescue the TS phenotype. Together these results demonstrate that expression of human Ku80 is not sufficient to rescue the TS phenotype of a HDF2 deficient strain, however, expression of the human Ku heterodimer can partially rescue this phenotype.

Expression of a human Ku heterodimer lacking the extreme N-terminus of Ku70 compromises the partial rescue of the TS phenotype of the HDF2 deficient yeast strain

It had been previously reported that removal of the extreme N-terminus of Ku70 impaired the ability of the Ku heterodimer to repair DNA and bind to DNA ends *in vitro* (Jin and Weaver, 1997). Also, I determined that a similar Ku variant lacking amino acids 1-57 of Ku70 was impaired for pleiotrophic activation of reporter genes in yeast. I was therefore interested in determining the effect of this deletion on the ability of Ku to partially rescue the TS phenotype of the HDF2 deficient yeast. I transformed the strain to express ADKu70/Ku80 or ADKu70$_{58-609}$/Ku80 and assessed for colony formation at 30 and 37 °C. The yeast transformed to express the WT Ku heterodimer formed colonies at

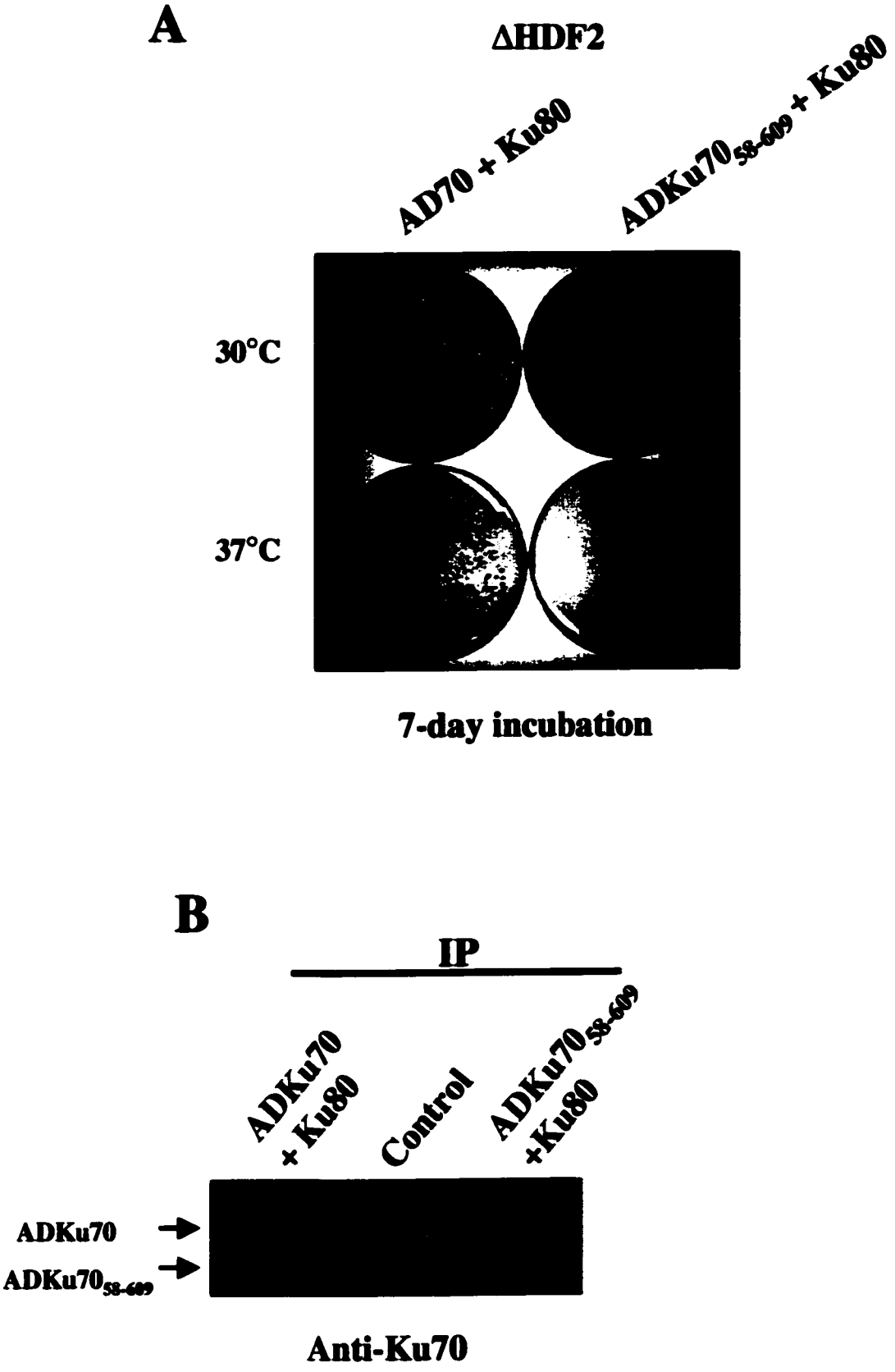
both the permissive and the restrictive temperatures, however the yeast transformed to express ADKu70$_{58\text{-}609}$/Ku80 formed colonies only at the permissive temperature (Fig. 13A).

To verify that the lack of rescue of the TS phenotype was not do to lower expression of the Ku deletion mutant, expression levels of the Ku heterodimers formed in the yeast were determined by immunoprecipitation with antibody 162. Antibody 162 is a monoclonal antibody specific for the human Ku heterodimer and does not react with the individual Ku subunits. Immunoprecipitation of Ku heterodimers with antibody 162 revealed that ADKu70$_{58\text{-}609}$/Ku80 and ADKu70/Ku80 accumulated to the same extent reflected by the similar amounts of ADKu70 proteins immunoprecipitated from the extracts (Fig. 13B). This data strongly suggests that the N-terminus of Ku70 is required for the human Ku heterodimer to partially rescue the TS phenotype of a HDF2 deficient yeast strain.

Expression of human Ku heterodimer does not rescue the slow growth phenotype of the HDF2 deficient yeast strain

In addition to the characteristic temperature sensitive phenotype of Ku deficient yeast, yeast lacking Ku also display a slow growth phenotype. The slower growth of the yeast is reflected by less yeast visible on a plate following incubation at 30 °C when compared to the parent strain. Also, this slow growth results in yeast colonies being smaller than those of the WT strain. To determine whether expression of the human Ku heterodimer could rescue the growth phenotype of yeast lacking HDF2, I compared the

Figure 13. The extreme N-terminus of Ku70 is required for the partial rescue of the temperature sensitive phenotype of the HDF2 deficient *Saccharomyces cerevisiae*. (A) The YMpLacZNRE1ΔHDF2 strain (ΔHDF2) lacking HDF2 was transformed to express ADKu70 or ADKu70$_{58-609}$ with Ku80 and colonies were selected on the appropriate synthetic dropout solid medium. A colony from each transformation was harvested and grown in liquid culture to mid-log phase. Yeast were then plated on medium and grown at 30 or 37 °C for 7 days. (B) Immunoprecipitation of Ku heterodimers. Heterodimer-specific antibody 162 was used to immunoprecipitate ADKu70/Ku80 or ADKu70$_{58-609}$/Ku80 from a yeast extract derived from the YMpLacZNRE1ΔHDF2 strain transformed to express these Ku heterodimers. An extract from yeast transformed with empty parent plasmids was included as a control (control). Immunoprecipitates were subjected to Western blot analysis and ADKu70 proteins were detected with antibody N3H10.


A
ΔHDF2
AD70 + Ku80
ADKu70$_{58-609}$ + Ku80
30°C
37°C
7-day incubation
B
IP
ADKu70 + Ku80
Control
ADKu70$_{58-609}$ +Ku80
ADKu70
ADKu70$_{58-609}$
Anti-Ku70

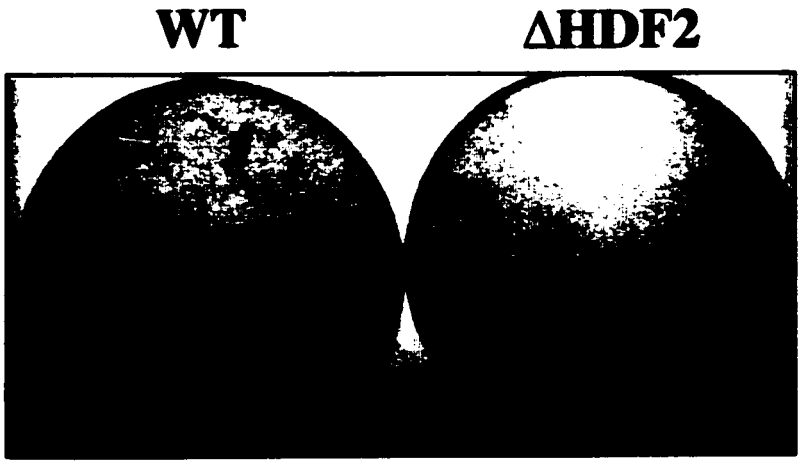
growth rate of my HDF2 deficient strain when transformed to express the human Ku heterodimer or with empty parent plasmids.

Equal amounts of the parent (WT) and HDF2 mutant (ΔHDF2) strains were plated and grown for 3 days at 30°C. Consistent with previous reports, the strain lacking HDF2 grew significantly slower than the parent strain reflected by less yeast visible on the plate (Fig. 14A). In contrast to the TS phenotype, co-expression of ADKu70 and Ku80 failed to rescue the slow growth of this strain. In fact, expression of ADKu70/Ku80 or ADKu70 alone resulted in even slower growth of the HDF2 deficient yeast at 30 °C compared to the yeast when transformed with the empty parent vectors. This more pronounced growth retardation was seen by significantly smaller colonies for the yeast expressing ADKu70/Ku80 or ADKu70 than the yeast transformed with the parent plasmids (Fig. 14B).

The slower growth observed for the yeast expressing Ku most likely reflects transcriptional squelching due to high levels of the Gal4 activation domain present in the yeast fused to Ku70. Transformation with the empty parent plasmids expressing the Gal4AD alone was not expected to show severe squelching effects as the Gal4AD when expressed alone accumulated to low levels in the yeast (data not shown). Further, this additional growth defect is not a unique property of Ku as I observed the same growth retardation with other constructs fused to the Gal4AD that were expressed at high levels in the yeast, namely the yeast TATA binding protein (data not shown). Nevertheless, this slower growth demonstrated clearly that the HDF2 deficient yeast expressing human Ku grow slower at 30 °C than when transformed with the parent plasmids. At 37 °C this strain grew better when transformed to express this same Ku (as yeast colonies were

Figure 14. Expression of the human Ku heterodimer does not rescue growth of the HDF2 deficient *Saccharomyces cerevisiae*. (A) The parent (WT) and HDF2 deficient strain (ΔHDF2) were grown in liquid culture to mid-log phase before plating an equal amount of yeast onto YPD medium and incubating at 30 °C. Shown are photographs of the plates after 3 days of incubation showing the slower growth rate of the ΔHDF2 strain. (B) The ΔHDF2 strain was transformed with plasmids expressing ADKu70 and Ku80, ADKu70 alone or with empty parent plasmids. Colonies were selected on solid synthetic dropout medium and one colony from each transformation was harvested and grown in liquid culture at 30 °C to mid-log phase. An equal amount of yeast was then plated onto appropriate medium for each transformation and incubated at 30 °C for 4 days to allow colonies to form. Shown is a photograph of the yeast colonies after the 4-day incubation. Yeast were transformed as follows: A, empty parent plasmids; B, ADKu70 + Ku80; C, ADKu70.

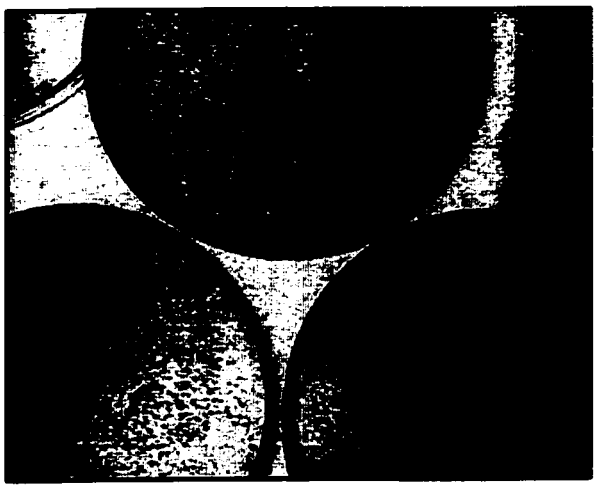
A



Yeast Growth at 30 °C

B

ΔHDF2



Yeast Growth at 30 °C

formed) than when transformed with the parent plasmids. These results suggest that the TS phenotype and the slow growth at 30 °C of HDF2 deficient yeast reflect deficiencies in two distinct cellular functions of Ku. The TS phenotype can be partially rescued by expression of the human Ku heterodimer and this same Ku fails to rescue the slow growth phenotype.

Part I: Discussion

To investigate the sequence-specific binding of Ku to NRE1 *in vivo* in the context of a chromatin environment I set up a yeast one-hybrid system. A yeast strain was constructed that contained the *lacZ* reporter with four tandem copies of NRE1 as the UAS integrated into the genome. This strain was then transformed to express Ku fused to the Gal4AD. Ku binding to NRE1 was expected to result in recruitment of the Gal4AD to the *lacZ* reporter gene and stimulate transcription. Therefore, Ku binding to NRE1 could be monitored by activation of *lacZ*. Surprisingly, expression of Ku fused to the Gal4AD resulted in *lacZ* activation independent of a UAS impeding the analysis of Ku binding to NRE1.

I discovered that human Ku when expressed in yeast fused to an ectopic activation domain pleiotrophically activated reporter genes integrated into the yeast genome. The activation was determined to be dependent on the Gal4 activation domain as expression of Ku without an activation domain failed to induce reporter gene expression. By expressing the individual Ku subunits I determined that the Ku70 monomer was sufficient for activation. Further, the level of ADKu70-dependent transcriptional induction of reporter genes was unaffected when expressing ADKu70 in a yeast strain in which I deleted the gene encoding for the yeast Ku80 homologue (HDF2) demonstrating that the observed activation was independent of HDF2. However, the transcriptional stimulation was not restricted to the Ku70 monomer as expression of Ku70 in combination with ADKu80 also activated reporter genes. Since expression of ADKu80 alone did not result in detectable stimulation of reporters, this observation

strongly suggested that the Ku heterodimer also had the ability to broadly activate reporter genes in yeast.

In addition, co-expression of ADKu70 with Ku80 significantly increased the level of reporter gene induction observed. This result was consistent with the elevated protein levels of ADKu70 detected in yeast that reflects stabilization of the ADKu70 monomer through dimerization with Ku80. Since the Ku subunits are tightly associated in the cell this increase in the level of transcription when expressing ADKu70 in combination with Ku80 most likely reflects activation predominately mediated by the ADKu70/Ku80 heterodimer.

Dissection of the components of Ku required for the ADKu-dependent activation revealed that both the N and C-terminus of the Ku70 monomer were required as expression of solely the N or C-terminal portions of Ku70 failed to induce reporter transcription. Interestingly, however, expression of an ADKu70 mutant comprised of amino acids 1-491 of Ku70 activated transcription only when assembled with Ku80. In this instance, it appears that Ku80 can functionally replace the C-terminus of Ku70 when assembled into a heterodimer to restore the activation property of a Ku70 mutant lacking its C-terminus. By contrast, internal deletions of ADKu70 demonstrated that internal portions of Ku70 were dispensable for activation, as expression of a mutant lacking amino acids 177-492 activated transcription to levels comparable to the ADKu70/Ku80 heterodimer. This stronger activation by this Ku70 internal deletion mutant was consistent with its higher expression in yeast.

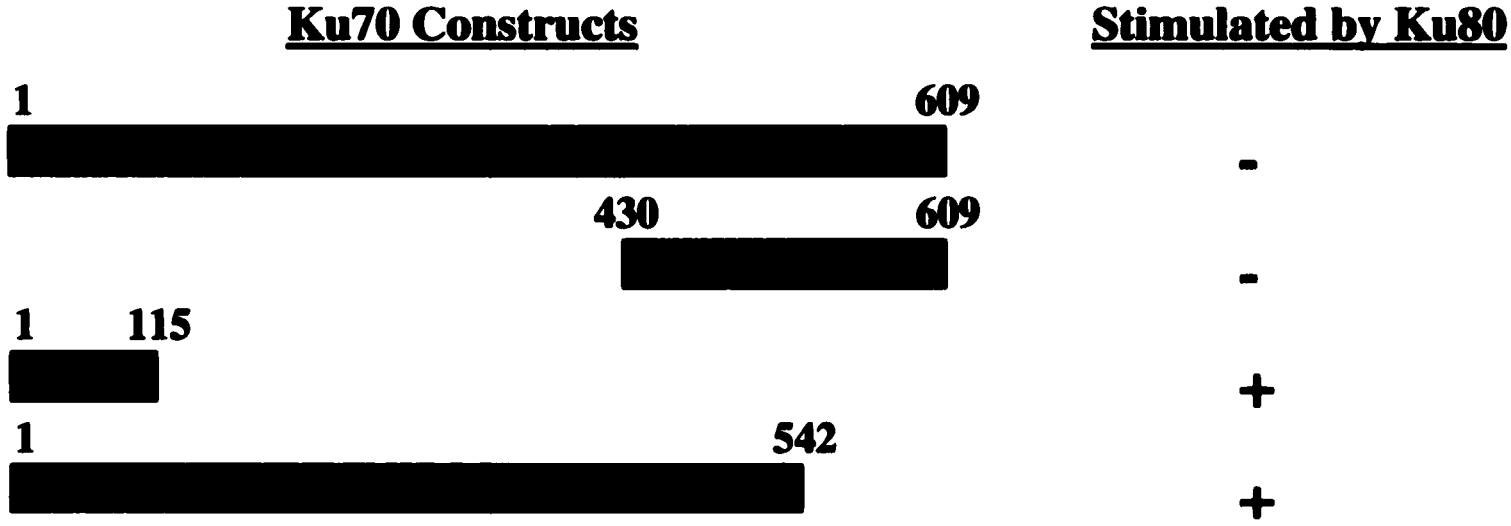
The observation that expression of the Gal4AD alone or fused to many other proteins (e.g. several human Ku deletion mutants, yeast TBP, HDF1) did not activate the

reporter genes used in this study demonstrates that accumulation of the Gal4AD in yeast in not sufficient to induce reporter expression. In addition, since expression of Ku without the Gal4AD also failed to activate reporter genes indicates that Ku was not sufficient. Sequestration of possible gene repressors by Ku was an unlikely explanation for the observed effect, as this mechanism would be expected to be independent of the Gal4AD. Together, these results indicate that determinants from Ku and the Gal4 activation domain are required for activation. Therefore, it is very likely that the transcriptional stimulation observed resulted from Ku-mediated recruitment of the Gal4AD to the yeast reporter genes and subsequent induction of transcription by the Gal4AD.

One explanation for the observed activation is that Ku recruits the AD to the yeast minimal promoters of the reporter genes through direct recognition of a DNA structure common to all the promoters of the reporter genes tested. In my analysis of the regions of Ku required for activation I found that they shared a striking similarity to portions of Ku previously identified to bind DNA ends *in vitro*. Figure 15 schematically illustrates regions of the Ku70 monomer that have been shown to bind to DNA ends in DNA immunoprecipitation assays (Wang et al., 1998a). Also indicated is whether dimerization with Ku80 increases the ability of the Ku70 fragments to bind DNA.

It has been reported that the full-length Ku70 can bind to DNA in the absence of Ku80 in both DNA immunoprecipitation and Southwestern experiments (Allaway et al., 1990; Chou et al., 1992; Mimori and Hardin, 1986; Wang et al., 1998a). Similarly, amino acids 430-609 of Ku70 also binds to DNA in the absence of assembly with Ku80 (Wang et al., 1998a). By contrast, the extreme N-terminus of Ku70, amino acids 1-115,

Figure 15. Regions of Ku70 that bind nonspecifically to DNA *in vitro* and effects of dimerization with Ku80. Shown is a schematic illustration of the amino acids of Ku70 deletion mutants that have been shown to bind nonspecifically to linear double-stranded DNA in DNA immunoprecipitation experiments. Also indicated is whether dimerization of the Ku70 proteins with Ku80 stimulated (+) or did not effect (-) the ability of the Ku70 fragments to bind DNA.


Ku70 Constructs
Stimulated by Ku80
1
609
-
430
609
-
1
115
+
1
542
+

does not bind efficiently to DNA in DNA immunoprecipitation assays in the absence of dimerization with Ku80 (Wang et al., 1998a). However, assembly of this portion of Ku70 with Ku80 resulted in a Ku heterodimer with increased ability to bind DNA (Wang et al., 1998a). Whether this increase in DNA binding of amino acids 1-115 of Ku70 requires Ku80 to fold properly or whether Ku80 participates directly in DNA binding is not known. Moreover, in this same assay it was shown that dimerization of a C-terminal Ku70 mutant comprised of amino acids 1-542 with Ku80 also significantly enhanced its ability to bind nonspecifically to DNA (Wang et al., 1998a). A different report showed that a Ku heterodimer lacking amino acids 1-62 of Ku70 was deficient in DNA repair and binding to DNA in EMSAs (Jin and Weaver, 1997). In my experiments removing amino acids 1-57 of Ku70 to form the ADKu70$_{58-609}$/Ku80 heterodimer abrogated the ADKu-dependent stimulation of reporter gene transcription in yeast. Further, dimerization of a Ku70 C-terminal mutant, ADKu70$_{1-491}$, with Ku80 restored the transcriptional activation property of Ku. Also, both the N and C-terminus of the Ku70 monomer were required for activation. The similarity in regions of Ku necessary for activation with portions of Ku needed to bind in a sequence-independent manner to DNA *in vitro* supports Ku binding to DNA as mediating the Ku-dependent activation in yeast.

Additional support that the Ku-dependent pleiotrophic activation results from Ku binding to DNA stems from biochemical fractionation experiments. I observed that transiently expressed Ku70 monomers are tightly associated with the nucleus in mammalian cells to a similar degree as the endogenous Ku heterodimer from HeLa cells. However, deletion of amino acids 1-57 of Ku70 that disrupted ADKu70-dependent reporter gene activation in yeast cells resulted in a Ku70 monomer that was less tightly

associated with the nucleus. Significantly more $Ku70_{58-609}$ than WT Ku70 was detected in the cytoplasmic fraction upon hypotonic lysis and biochemical fractionation of the cells. Approximately 50 % of $Ku70_{58-609}$ was detected in the cytoplasmic fraction as opposed to approximately 10 % for the full-length Ku70. The larger proportion of $Ku70_{58-609}$ found in the cytoplasmic fraction indicates that more of this Ku mutant is freely circulating in the nucleus and can be eluted from the nucleus upon disruption of the plasma membrane and dilution from the nucleus with hypotonic buffer. Detection of more $Ku70_{58-609}$ in the cytosolic fraction likely reflects impaired DNA binding by the Ku70 monomer. Consistent with this is the observation that for many transcription factors and other proteins, the loss of DNA binding *in vivo* is reflected by the loss of these proteins from the nucleus upon lysis of the cell in a hypotonic buffer that results in pronounced swelling of the nucleus.

The larger proportion of $Ku70_{58-609}$ in the cytoplasmic fraction compared to the WT Ku70 is not a consequence of differences in nuclear accumulation as both constructs are efficiently targeted to the nucleus and are completely nuclear in immunofluorescence experiments. I show in the next section of my thesis that both these constructs contain the Ku70 nuclear localization signal and N-terminal deletions of Ku70 to amino acid 495 has no effect on the nuclear accumulation of Ku70. Also, our lab has shown that this exact construct, $Ku70_{58-609}$, is completely nuclear by indirect immunofluorescence as the WT Ku70 in XR-V15B cells.

Our lab has now gone forth to show that both the Ku heterodimer and Ku70 monomer is efficiently released from mammalian nuclei upon DNaseI treatment of the nuclei (Bertinato et. al. manuscript in preparation). The efficient release of the Ku

heterodimer and Ku70 monomer with limiting concentrations of DNaseI, that digests preferentially euchromatin and transcriptionally active DNA, is further evidence that a significant proportion of Ku is associated with relaxed and transcriptionally active chromatin in mammalian cells.

Since the DNA sequences flanking the yeast minimal promoters of the different reporter genes that were activated by Ku share little sequence homology, it is unlikely that the Ku mediated transcriptional activation resulted from Ku binding directly to a specific DNA sequence element. Further, there are no DNA sequences in the vicinity of the yeast minimal promoters that closely resemble NRE1 and the transcriptional activation of the reporter gene containing the sequence-specific binding site for Ku, NRE1, as the UAS was not activated more strongly by Ku in my assays. Therefore, if the Ku-dependent activation resulted through Ku binding directly to DNA, it is most likely that Ku was recognizing DNA in a sequence-independent manner and binding to a common DNA structure present at all the reporter genes analyzed.

The Ku-dependent activation may be explained by Ku interacting directly with structured DNA resulting from the more open DNA of yeast promoters compared to DNA of coding regions. In eukaryotes, the wrapping of DNA around an octamer of histones to form nucleosomes restricts access of the DNA to transcription factors such as the TATA box binding protein, activator proteins and RNA polymerase II by masking the DNA target sites. Several complexes have now been identified that function to alter chromatin structure making the DNA wrapped around nucleosomes more assessable to recognition by DNA binding proteins (Kornberg and Lorch, 1999; Travers, 1999; Vignali et al., 2000). By contrast, complexes exist that modify the chromatin structure in a way

that further restricts access to the DNA by promoting a more compact chromatin structure (Kornberg and Lorch, 1999). DNA unwinding downstream from the initiation start site in the preparation for transcription is well documented (Larsen and Weintraub, 1982; Nickol and Felsenfeld, 1983). In addition to the unwinding of DNA downstream of the initiation start site it has been reported that regions of DNA upstream of the DNA polymerase binding site at specific promoter regions also undergo conformational changes that can be detected by single strand specific nucleases (Giardina and Lis, 1993; Giardina and Lis, 1995).

Analysis of yeast promoter DNA regions using probes of chromatin structure has established that DNA of many yeast promoters, including the *CYC1*, *HIS3* and *GAL1* yeast promoters used in my study, adopt a DNA conformation that is more accessible to DNA binding proteins (Giardina and Lis, 1993; Giardina and Lis, 1995; Mai et al., 2000; Yagil et al., 1998). DNA promoter regions have been shown to contain transcription-independent regions of nuclease hypersensitivity and experiments using micrococcal-nuclease suggest that the hypersensitivity observed reflect either nucleosome free DNA or at least structured DNA (Erkine et al., 1995; Fascher et al., 1993; Lee and Garrard, 1992; Lorch et al., 1998). Furthermore, it was reported that the Ty1 retrotransposon preferentially integrates in promoter rather than coding DNA regions in yeast, again supporting other data that indicates DNA of yeast promoters is more assessable (Eibel and Philippsen, 1984; Natsoulis et al., 1989).

The exact feature of yeast promoters that promotes a more open structure of the chromatin is not completely understood. One possibility is that it is due to an intrinsic property of the DNA sequence. It is now known that yeast promoter DNA sequences are

A, T rich. Whole genome analysis revealed that poly(dA-dT) tracts are abundant in *S. cerevisiae* and occur mainly at unit nucleosomal length upstream and downstream of open reading frames (Raghavan et al., 1997). This observation lead to the proposal that such tracts modulate nucleosome positioning. In support of this conclusion are experiments that have shown that a poly(dA-dT) element located adjacent to the *Candida glabrata* metal-dependent transcriptional activator gene, *AMT1*, functions in transcriptional autoactivation by causing a localized distortion of nucleosomal DNA, allowing Amt1 to bind its promoter element (Zhu and Thiele, 1996). Also, it has been proposed that A, T rich DNA regions constitute the classical DNA unwinding motif (Umek and Kowalski, 1988). Both the *CYC1* and *HIS3* promoters used in this study that were activated by Ku contain regions of A, T richness.

Another feature shared by yeast promoters that may account for a more open conformation of the DNA is that many promoters contain DNA regions that are entirely or mainly homopurine on one strand and homopyrimidine on the complementary strand. These oligopurine.oligopyrimidine (R.Y) tracts are also present on many other promoter regions in higher eukaryotes and these regions of promoters have been shown to be sensitive to single strand specific nucleases suggesting that the DNA of these sequences can form structures (Glaser et al., 1990; Larsen and Weintraub, 1982; Wells et al., 1988; Yagil, 1991). Recently, it was reported that the *CYC1* promoter, that contains long R.Y. tracts, when in the context of a negatively supercoiled plasmid undergoes a transition into an unwound state that resulted in extensive cleavage within the R.Y. tracts by the single strand specific nuclease P1 (Yagil et al., 1998). These results suggest that at sufficient torsional stress, the R.Y. tracts can undergo a transition into an unwound state and may

serve as DNA unwinding centers in gene promoters. The *CYC1* and *GAL1* promoters used in this study also contain oligopurine.oligopyrimidine stretches.

Recently, it has been suggested that preferential accessibility of yeast promoter regions does not depend on a specific sequence element. It was postulated that preferential accessibility of yeast promoter DNA is through multiple determinants within the promoter region that contribute to its more accessible structure (Mai et al., 2000). It was suggested that each determinant adds a specific component to increase the accessibility to the promoter DNA. This was demonstrated by progressive deletion of the *HIS3* promoter from one end and then the other end that resulted in increasing loss of accessibility to a restriction endonuclease. These results suggest that the more open structure may reflect positioning of the nucleosome cores by certain sequence periodicities that are related to DNA bending. For example, the minor grooves of AAA and AAT face inward towards histones, whereas those of GGC and AGC face outward (Drew and Travers, 1985; Satchwell et al., 1986). Therefore, perhaps these periodicities of di and tri nucleotides in yeast promoters together promote a unique DNA structure that is attractive to Ku. Since Ku binds with high affinity to virtually any double-to-single stranded transition of DNA *in vitro* it is possible that any DNA structure presented by the chromatin of yeast promoters resembling a double-to-single stranded DNA transition would be recognized by Ku.

An important feature of the reporter genes used in my study is that the promoters that were induced by Ku were all minimal promoters that represent a portion of the WT promoter. However, the same region of the *HIS3* minimal promoter that was stimulated by ADKu was shown to be approximately 5 times more accessible to cleavage by the

restriction endonuclease HinfI compared to coding DNA sequences *in vivo* (Mai et al., 2000). This result indicates that even short DNA segments representing the minimal promoter region of yeast promoters can have sufficient determinants that allow the DNA to assume a more accessible conformation that is more readily recognized by DNA binding proteins.

Although the Ku-dependent activation was broadly observed, I noticed that ADKu70/Ku80 more potently induced expression of the p53-*CYC1-lacZ* reporter than the p53-*HIS3-HIS3* reporter when comparing reporter gene activation levels to those induced by ADp53. I found that ADKu70/Ku80 activated the p53-*CYC1-lacZ* reporter gene to levels comparable to ADp53 whereas ADp53 activated the p53-*HIS3-HIS3* reporter much more strongly. A possible explanation for the stronger activation of the p53-*CYC1-lacZ* reporter by Ku is that the *CYC1* promoter adopts a more pronounced open DNA structure that is more readily recognized by Ku than the *HIS3* promoter. This more accessible configuration of the *CYC1* promoter DNA region is supported by reports that have shown constitutive occupancy of both TBP and RNA polymerase II at the promoter even when transcriptionally silent (Kuras and Struhl, 1999). Further, the *CYC1* promoter has been shown to adopt a partly unwound conformation when co-crystallized with TBP (Kim et al., 1993a; Kim et al., 1993b). The stronger activation of the p53-*CYC1-lacZ* gene would be consistent with Ku recognizing a more open or active chromatin configuration. Nonetheless, it is quite remarkable that for the p53-*CYC1-lacZ* reporter ADKu70/Ku80 appears to activate transcription to similar levels as ADp53 without any apparent sequence-specific binding of Ku.

While human ADKu70 globally activated reporter genes in yeast no activation was detected when expressing the yeast Ku70 homologue (HDF1) fused to the Gal4 activation domain despite similar protein expression levels of the two constructs in yeast. ADHDF1 failed to activate any of the reporters tested. Furthermore, my results indicate that it is also unlikely that the yeast Ku heterodimer possesses the activation property as ADHDF1 failed to activate reporters in yeast strains that express endogenous HDF2 where it is expected that the ADHDF1/HDF2 heterodimer would form normally.

The absence of activation by the yeast Ku may reflect distinct differences in the DNA binding modes of the yeast and human Ku. In my experiments I found that the extreme N-terminus of Ku70 was required for activation. Significantly, this region is not well conserved in the yeast Ku70 homologue. Furthermore, the SAP domain, a putative DNA binding domain found in the C-terminus of human Ku70 and Ku70 from other higher eukaryotes is poorly conserved in HDF1 possibly suggesting a difference in the DNA binding properties between human and yeast Ku. Differences in the manner in which human and yeast Ku bind to DNA would not be completely surprising, as the predominant DNA repair pathways in yeast and higher eukaryotes are different. If the Ku-dependent activation does reflect human Ku binding to DNA it would be interesting to compare human and yeast Ku binding to different forms of DNA such as DNA ends, DNA structures and specific sequences. The modes in which the human and yeast Ku bind to DNA would be expected to differ. These experiments would provide important clues on how Ku binding to DNA mediates its multiple activities in the cell.

An interesting observation of the Ku-dependent activation was that the ADKu70 monomer appeared to activate transcription to similar levels as the ADKu70/Ku80

heterodimer when adjusting for the amount of ADKu70 present in the yeast. This was also supported by the observation that the ADKu70 internal deletion mutant lacking amino acids 177-492 activated transcription to levels comparable to the Ku heterodimer consistent with its higher level of expression. These results may imply that in the intact cell in the context of chromatin the Ku70 monomer binds to DNA (associates with chromatin) with similar affinity as the Ku heterodimer. The potential higher affinity binding of the Ku70 monomer to DNA than what has been reported using *in vitro* experiments may simply be a reflection of *in vivo* verses *in vitro* conditions. Alternatively, Ku70 may interact with higher affinity with a particular structure presented by the chromatin that is considerably different than the DNA used in the *in vitro* experiments.

If the Ku-dependent activation observed is mediated by Ku binding to DNA my data would suggest an alternative mode for Ku binding to DNA that requires the extreme N-terminus of Ku70. For Ku70 binding nonspecifically to DNA in DNA immunoprecipitation assays and Southwestern experiments the C-terminus of Ku70 that contains the putative DNA binding SAP motif seems to be the major determinant. This is supported by findings that show that the C-terminus of Ku70 binds to DNA with a similar affinity to the full-length Ku70 in DNA immunoprecipitation experiments (Wang et al., 1998a). However, in my experiments I observed no activation by expressing a large portion of the C-terminus of Ku70 either alone or when assembled with Ku80. Also, for the activation property of ADKu70 the C-terminus of Ku70 is not essential as it can be replaced by dimerization with Ku80. Therefore, if the Ku-dependent activation of reporter genes in yeast is through Ku binding to a DNA structure, my results would

suggest that for Ku to interact with chromatin of yeast *in vivo* a different mode of binding is likely that depends mostly on the extreme N-terminus of Ku70.

The finding that human Ku fused to an ectopic activation domain pleiotrophically activates reporter genes in yeast and that this property may reflect Ku binding to a unique DNA structure presented by yeast promoter DNA regions might contribute to an explanation of a number of reported functions for Ku in higher eukaryotes. One possibility is that Ku may participate in the regulation of transcription of specific genes in higher eukaryotes that contain specific DNA sequence elements that when assembled into chromatin induce a more open DNA conformation that Ku can bind. It has been reported that overexpression of Ku in rodent cells specifically represses induction of the hsp70 gene upon heat shock (Li et al., 1995). Similarly, overexpression of Ku in K562 cells repressed a reporter gene under control of the gycophorin B promoter (Camara-Clayette et al., 1999). Interestingly, in these experiments it appeared that Ku70 could function independently of Ku80 consistent with the ADKu70 monomer being sufficient to activate reporter genes in yeast.

Another possibility is that Ku may play a more general role in the regulation of gene transcription. Recently, it has been reported that cells lacking Ku are deficient in transcriptional reinitiation and it has been suggested that Ku mediates recruitment of a soluble factor that is limiting for reinitiation (Woodard et al., 2001). Further, Ku has also been shown to fractionate with the RNA polymerase II holoenzyme and interact with TBP (Dvir et al., 1992; Genersch et al., 1995). I have also shown that Ku interacts with TBP (Appendix A). Furthermore, phosphoryation of TBP and TFIIB by Ku/DNA-PKcs was shown to synergistically stimulate basal transcription *in vitro* (Chibazakura et al.,

1997). In this instance, it is possible that Ku bound to active chromatin may recruit and stimulate DNA-PKcs activity resulting in the phosphorylation of specific transcription factors.

An alternative possibility is that the Ku-dependent activation reflects a structural role for Ku in the regulation of transcription in higher eukaryotes. In eukaryotes, RNA polymerase II mediated transcriptional activation by activator proteins bound to an upstream activating sequence can occur by interactions between the activator protein and TFIID or the RNA polymerase II holoenzyme. Alternatively, activation by an activator can be indirect through interactions with co-activators that interact with factors of the core transcriptional machinery or with co-activators that modify chromatin structure. Ku may function as a structural protein at transcriptionally active chromatin facilitating protein-protein interactions by maintaining the chromatin in a favorable conformation. Consistent with a structural role for Ku is a report suggesting that Ku can bind to the base unpairing regions (BURs) of matrix attachment regions (Galande and Kohwi-Shigematsu, 1999; Galande and Kohwi-Shigematsu, 2000). Further, Ku has also been implicated in maintaining the integrity of the genome by suppressing chromosomal rearrangements (Difilippantonio et al., 2000).

The recruitment of Ku to yeast promoters may also reflect a role for Ku in NNEJ. Recently, phosphorylation of a conserved motif in histone H2A has been implicated in double-stranded DNA break repair in *S. cerevisiae* (Downs et al., 2000). It was postulated that phosphorylation of a core histone in response to DNA damage may alter the conformation of the chromatin in a manner that facilitates DNA repair (Downs et al., 2000). Although, the DNA repair pathway in which histone H2A appears to participate

does not seem to be epistatic with Ku in yeast, it is possible that in higher eukaryotes Ku may function in a similar DNA repair pathway.

If the Ku-dependent stimulation of reporter genes in yeast is not a result of Ku binding directly to a DNA structure, an alternative possibility is that Ku may interact with a common endogenous yeast factor tethered to DNA. However, this factor would have to be present at all the reporters examined. Since the promoters driving expression of the reporter genes activated by Ku were minimal promoters many of the specific sites for DNA binding factors that are normally present on the WT promoters are absent. In addition, chromatin immunoprecipitation assays have shown that at least for the *GAL1* promoter transcription factors such as TBP and RNA polymerase II are not associated with the promoter DNA in significant amounts when transcriptionally inactive, as was the situation in my experiments (Kuras and Struhl, 1999). However, Ku may interact with histones or specifically modified histones. For example, Ku may interact with histones that have been acetylated by chromatin remodeling complexes or phosphorylated at particular sites by specific kinases. However, to date there is no evidence for an interaction of Ku with histones.

Ku has been highly conserved throughout evolution. This is underscored by observations that human Ku70 and Ku80 can complement Ku deficient phenotypes in other species. In particular, it was reported that expression of human Ku70 in a yeast strain deleted for the *HDF1* gene complemented the temperature sensitive phenotype suggesting that human Ku70 can functionally substitute for HDF1 to rescue the TS phenotype (Barnes and Rio, 1997). However, I determined that human Ku80 does not rescue the TS phenotype of a yeast strain deleted for HDF2 as colonies failed to form

when the yeast were incubated at 37 °C. The absence of complementation may reflect a defect in the dimerization between HDF1 and human Ku80 as it has been previously reported that extracts from yeast co-expressing HDF1 and human Ku80 do not display Ku DNA binding activity (Feldmann et al., 1996). These results indicate that the TS phenotype reflects a more important or species specific role for HDF2. Also, this data shows that Ku70 is more functionally conserved than Ku80 between yeast and humans in the Ku-dependent pathway that results in the TS phenotype.

Interestingly, co-expression of both human ADKu70 and Ku80 resulted in a partial rescue of the TS phenotype of the HDF2 deficient strain as yeast colonies formed at the restrictive temperature. However, upon streaking, the yeast failed to continue to grow at the nonpermissive temperature. An interpretation of these results is that the human Ku heterodimer can partially function in a yeast Ku pathway to partially overcome the TS phenotype.

In my experiments I did not observe colony formation for the HDF2 deficient yeast at the restrictive temperature. For HDF1 deficient yeast, it was reported that colonies do form at the restrictive temperature but fail to continue growing upon restreaking and further incubation at 37 °C (Barnes and Rio, 1997). This result may suggest a more important role for HDF2 in the pathway that results in the TS phenotype. However, since the yeast strains in the two studies were different and there may be subtle variations in the experimental conditions used caution is suggested when interpreting these results.

I determined that a Ku heterodimer lacking amino acids 1-57 of the N-terminus of Ku70 failed to pleiotrophically activate yeast reporter genes. Interestingly, when

expressing this Ku variant in the HDF2 deficient yeast strain I observed that it was insufficient to partially rescue the TS phenotype despite being expressed to similar levels as the full-length Ku. A similar variant of the Ku heterodimer lacking amino acids 1-61 of Ku70 was reported to be deficient in DNA binding and repair in mammalian cells (Jin and Weaver, 1997). These results are in agreement with other reports that suggests the yeast TS phenotype is a consequence of an accumulation of DNA damage in the cell ultimately resulting in death.

One possible explanation for the elevated temperature causing death due to an accumulation of DNA damage in yeast is that the increased temperature may inactivate or significantly reduce the function of a redundant DNA repair pathway that can function at the lower temperature. Another explanation is that at 37 °C the doubling time for yeast is shorter resulting in faster growth of the yeast compared to yeast growing at 30 °C. In the faster growing yeast a compensatory pathway may not have sufficient time to efficiently repair the entire damaged DNA.

Alternatively, it has also been proposed that the TS phenotype may result from a deficiency in Ku regulated chromosomal end structure (Gravel et al., 1998). It has been demonstrated that Ku is required for maintaining normal DNA end structure on yeast chromosomes (Gravel et al., 1998).

Unexpectedly, I observed that expression of the ADKu70/Ku80 heterodimer failed to rescue the slow growth phenotype of the HDF2 deficient strain at 30 °C. In fact, expression of ADKu70/Ku80 further retarded the yeast growth at the permissive temperature. This slower growth of the HDF2 deficient strain when transformed to express ADKu70/Ku80 most likely represents squelching due to the presence of high

levels of the Gal4 activation domain in the yeast rather than an effect attributed to expressing human Ku in the yeast. This is supported by observations that I have made with other Gal4AD fusion proteins that are expressed to high levels in yeast where I see a similar growth retardation at 30 °C. Moreover, I did not observe this slower growth of the HDF2 deficient strain when expressing the human Ku heterodimer without the Gal4 activation domain, which I determined to be expressed to the same level.

The partial rescue of the TS phenotype and not the growth phenotype of the HDF2 deficient yeast when expressing the same Ku heterodimer suggests that the TS and growth phenotypes represent to distinct functions of the yeast Ku. Further, it suggests that the TS phenotype reflects a more evolutionarily conserved function of Ku between yeast and humans. If the TS phenotype of yeast lacking Ku reflects a deficiency in DNA repair, than the growth phenotype must result in a defect in another distinct cellular process in which Ku participates.

One explanation for the slow growth phenotype is that Ku may function in a certain stage of mitosis. Evidence for a role for Ku in chromosome condensation has already been reported in mammals (Munoz et al., 1998). DNA topoisomerase II catalyzes topological changes in DNA that are crucial for normal cell cycle progression. Rodent cells lacking Ku80 display abnormal chromosome condensation in response to low doses of a DNA topoisomerase II inhibitor and accumulate in the G_2/M phase of the cell cycle (Munoz et al., 1998). This property was not observed in WT cells (Munoz et al., 1998). Further, these phenotypes in response to the inhibitor were not observed in cells deficient in DNA-PK activity suggesting this property of Ku is independent of DNA-PK mediated DNA repair (Munoz et al., 1998).

Therefore, the extended G_2/M phase of the cell cycle observed in Ku80 deficient cells (Nussenzweig et al., 1996) may be explained by Ku normally functioning together with topoisomerase II during mitosis. The absence of this activity of Ku in Ku80 deficient cells may translate to the slow growth phenotype. Moreover, it has been shown that yeast topoisomerase II associates with a helicase and both the topoisomerase and DNA helicase activities are necessary for chromosome segregation during mitosis (Watt et al., 1995). Interestingly, DNA helicase activity for Ku has already been reported (Ochem et al., 1997).

Part II: Introduction

Ku is predominately a nuclear protein that displays a diffuse nucleoplasmic distribution (Koike et al., 1999b; Koike et al., 1999c). However, Ku has also been reported to be localized to the nuclear periphery, to transcriptionally active regions and to be a component of the nuclear matrix (Higashiura et al., 1992; Yaneva and Jhiang, 1991; Yu et al., 1998). Also, after exposure of cells to irradiation, Ku was localized to discreet foci in the nucleus that may represent sites of DNA damage (Goedecke et al., 1999). Analysis of the subcellular localization of Ku during stages of the cell cycle revealed that the return of Ku70 into the nucleus as cells enter G_1 preceded the return of Ku80 in HeLa-S3 cells (Koike et al., 1999b). In addition to the nucleus, Ku has been detected in the cytoplasm in some cell types (Bakalkin et al., 1998a; Pucci et al., 2001; Tovari et al., 1998). Also, both Ku70 and Ku80 have been detected on the cell surface (Dalziel et al., 1992; Teoh et al., 1998). For Ku80, its localization on the plasma membrane was demonstrated to promote homotypic and heterotypic fusion of multiple myloma cells (Teoh et al., 1998). Furthermore, Ku80 has been proposed to be a somatostatin receptor and it was reported that a somatostatin analog induces translocation of Ku80 from the cytoplasm into the nucleus in colon tumour cells (Tovari et al., 1998).

The functions of many proteins are regulated by signals that determine their distribution within the cell. It is therefore important to understand the mechanisms by which proteins localize to specific regions in the cell in order to understand how they function. This is especially true for a protein such as Ku that acts at multiple subcellular compartments. The objective of my research was to characterize the sequences that

mediate the nuclear localization of Ku. These initial experiments will provide important information on the mechanism that targets Ku to the nucleus.

To delimit the import signals of Ku, I chose a transient transfection strategy using the XR-V15B cell line. XR-V15B cells are hamster ovarian fibroblasts that contain an inactivating mutation in the Ku80 gene. As a consequence of this mutation no Ku80 is detected in these cells. Further, these cells are depleted in Ku70 as the Ku70 monomer is unstable in the absence of dimerization with Ku80. Also, the endogenous hamster Ku70 levels rise only slowly after ectopic expression of Ku80. Therefore, these cells are ideal for the identification of the nuclear import signals of both Ku70 and Ku80 since it is expected that the endogenous Ku will not influence localization of the transfected Ku subunits.

To facilitate the mapping of the nuclear import sequences of Ku I chose to express the Ku subunits as EGFP (enhanced green fluorescent protein) fusions that will allow direct visualization of the distribution of the Ku subunits in live cells, as EGFP is naturally fluorescent. EGFP is a protein that has been extensively used to study the subcellular localization of recombinant proteins in cells. EGFP is ideal for these experiments as it does not contain subcellular targeting motifs and is not expected to influence the subcellular distribution of Ku fragments. The addition of the EGFP moiety provides the additional advantage of adding bulk to smaller Ku constructs that may otherwise be able to passively diffuse through the nuclear pore complex possibly resulting in ambiguity in the determination of their subcellular localization.

In this section I show that Ku70 and Ku80 each contain one basic NLS that is sufficient to localize the individual Ku subunits and the Ku heterodimer to the nucleus.

The import signal on Ku70 was determined to be composed of two basic clusters encompassing amino acids 539-556. The Ku80 NLS was identified as a short basic sequence comprising amino acids 565-569. Further, I show that inactivation of both NLSs results in a Ku heterodimer that is exclusively localized to the cytoplasm. Moreover, I show that point mutations in Ku80 previously reported to disrupt Ku heterodimer formation *in vitro* and in yeast two-hybrid experiments reduces the level of Ku70 without affecting heterodimerization of the Ku subunits in mammalian cells.

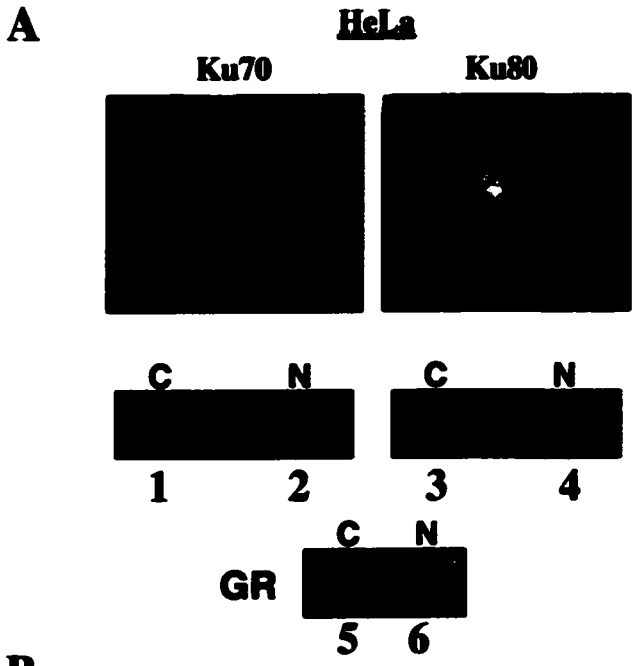
Part II: Results

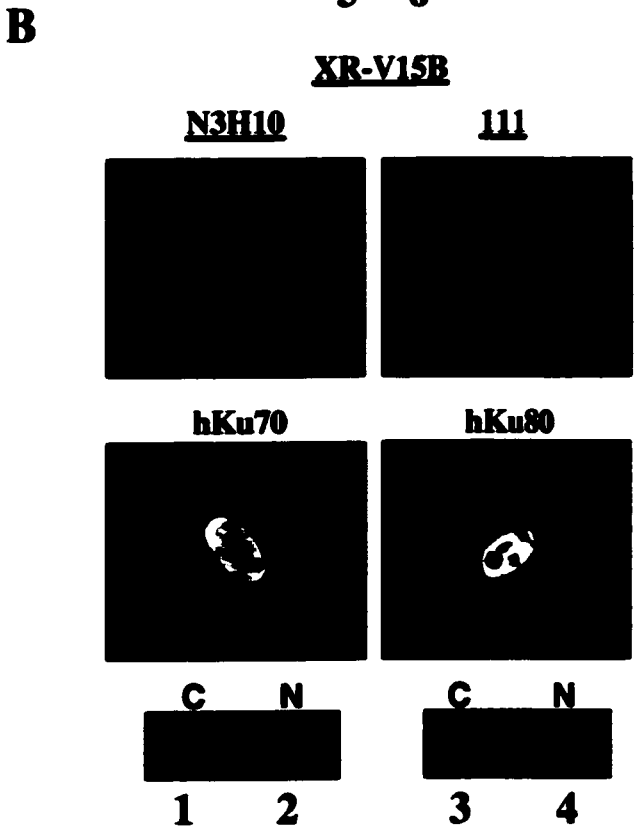
The individual Ku subunits localize to the nucleus

To begin the characterization of the signals that are required for the targeting of Ku to the nucleus, I established a transient expression system in XR-V15B hamster ovary fibroblast cells. In many cells types Ku is localized predominately in the nucleus, however in some tissues Ku has been shown to be distributed to other compartments including the cytoplasm and cell surface (Bakalkin et al., 1998a; Dalziel et al., 1992; Pucci et al., 2001; Teoh et al., 1998; Tovari et al., 1998). To ensure that my system would allow the identification of the nuclear import signals of Ku, I wanted to confirm that transiently expressed Ku in XR-V15B cells distributed to the nucleus as endogenous Ku from HeLa cells. This experiment would provide confidence that my transient transfection system accurately reflects the distribution of natural Ku in the cell.

I observed that Ku from HeLa cells was exclusively nuclear by indirect immunofluorescence and displayed a diffuse nucleoplasmic distribution (Fig. 16A). Also, I determined that the majority of the Ku70 and Ku80 subunits in HeLa cells were tightly associated with the nucleus and remained nuclear upon biochemical fractionation of the cells (Fig. 16A, lanes 2, 4) demonstrating that Ku was tightly associated with the nucleus. However, a small, but significant proportion of Ku70 and Ku80 was released from the nucleus upon fractionation (Fig. 16A, lanes 1, 3). Steroid treated glucocorticoid receptor (GR) transcription factor that associates tightly with DNA remained completely within the cell nucleus in the same assay (Fig. 16A, lanes 5, 6).

Figure 16. Localization of natural and recombinant Ku antigen determined by indirect immunofluorescence and subcellular fractionation. (A) HeLa cells. The localization of endogenous Ku was determined by indirect immunofluorescence (top) with Ku70-specific antibody N3H10 and Ku80-specific antibody 111. Localization of Ku subunits following subcellular fractionation was accomplished by Western blot of equally loaded SDS PAGE gels of nuclear (N) and cytosolic (C) fractions probed with the same antibodies (bottom, lanes 1-4). The distribution of steroid treated GR is shown as evidence of nuclear integrity following subcellular fractionation (lanes 5, 6). (B) XR-V15B cells. Indirect immunofluorescence using the Ku70 and Ku80-specific antibodies N3H10 and 111 was used to examine the localization of WT human Ku70 and Ku80 subunits expressed individually by transient transfection as indicated. The photomicrographs at the top were taken of untransfected cells, those at the bottom following transfection of Ku70 and Ku80 expression vectors, respectively. At the bottom are Western blots illustrating the distribution of Ku70 and Ku80 in nuclear (N) and cytosolic (C) fractions of the transfected cells (lanes 1-4). The scale bars within the photomicrographs represent 10 μm.


A
HeLa
Ku70
Ku80
C
N
C
N
1
2
3
4
C
N
GR
5
6


B
XR-V15B
N3H10
111
hKu70
hKu80
C
N
C
N
1
2
3
4

This control indicated that cytosolic Ku did not occur as a result of disruption of the nuclear envelope.

Ku exists in the cell mainly as a stable heterodimer of a Ku70 and Ku80 subunit. To facilitate the mapping of the nuclear import signals of the Ku heterodimer I chose to first identify the signals present on the individual Ku subunits. I began by examining the localization of Ku monomers expressed by transient transfection in XR-V15B cells cultured in the same media and under the same conditions as the HeLa cells employed above (Fig. 16B).

XR-V15B cells were originally selected by their hypersensitivity to ionizing radiation. It was determined that XR-V15B cells contain a specific inactivating mutation in the Ku80 gene. This mutation results in the absence of the Ku80 protein from these cells. As a consequence XR-V15B cells are also depleted in Ku70 due to the increased turnover of the Ku70 monomer in the absence of dimerization with Ku80. Furthermore, endogenous Ku70 levels in XR-V15B cells rise only slowly over several days following ectopic expression of Ku80. Therefore, it is expected that these cells will allow identification of the nuclear import signals of transiently expressed Ku70 and Ku80 at early times following transfection.

XR-V15B cells that were not transfected to express Ku70 and Ku80 showed no immunofluorescence using Ku70 and Ku80-specific antibodies N3H10 and 111, respectively (Fig. 16B, top). Indirect immunofluorescence analysis of XR-V15B cells with antibodies N3H10 and 111 respectively, 24 hours post transfection of Ku70 and Ku80 expression plasmids showed both Ku subunits to be exclusively nuclear proteins (Fig. 16B). Both Ku70 and Ku80 also remained mainly within the nuclear fraction upon

biochemical fractionation of the cells (Fig. 16B, lanes 1-4) suggesting that the majority of the Ku70 and Ku80 monomers are tightly associated with the nucleus as was the endogenous Ku heterodimer from HeLa cells. However, like the endogenous Ku from HeLa cells a small proportion of the individual Ku subunits were detected in the cytoplasmic fraction indicating that a small fraction of Ku is not bound in the nucleus and therefore released from the nucleus upon hypotonic lysis of the cells. Together, these results demonstrated that both Ku70 and Ku80 could localize to the nucleus independent of dimerization with their partner and that both Ku subunits associated with nuclear components in a manner that largely prevented their loss from the nucleus upon biochemical fractionation of the cells.

To ease the mapping of the nuclear import signals within each of the Ku subunits I chose to express Ku70 and Ku80 fused to the enhanced green fluorescent protein (EGFP). This would allow me to directly visualize the subcellular localization of Ku70 and Ku80 in live cells. To ensure that the addition of the EGFP moiety had no significant effect on the localization of either Ku subunit I expressed full-length Ku70 and Ku80 as EGFP fusions in XR-V15B cells and examined their cellular distribution by direct fluorescence. Both EGFPKu70 and EGFPKu80 were observed to be completely within the nucleus of live XR-V15B cells demonstrating that the EGFP tag did not effect the localization of the Ku subunits (Fig. 17). In contrast to EGFP-tagged Ku70 and Ku80, expression of EGFP alone was detected in both the nucleus and cytoplasm reflected by the uniform staining throughout the entire cell. This equal distribution of EGFP between the nucleus and cytoplasm is not surprising for a protein of

Figure 17. Distribution of EGFP-tagged Ku70 and Ku80 in XR-V15B cells. The localization of recombinant human Ku subunits fused to EGFP and EGFP alone as indicated was determined by direct fluorescent observation in live cells. Both EGFPKu70 (GKu70) and EGFPKu80 (GKu80) are localized to the nucleus whereas EGFP alone is distributed equally between the nucleus and cytoplasm. Bar, 10 μm.

XR-V15B Cells

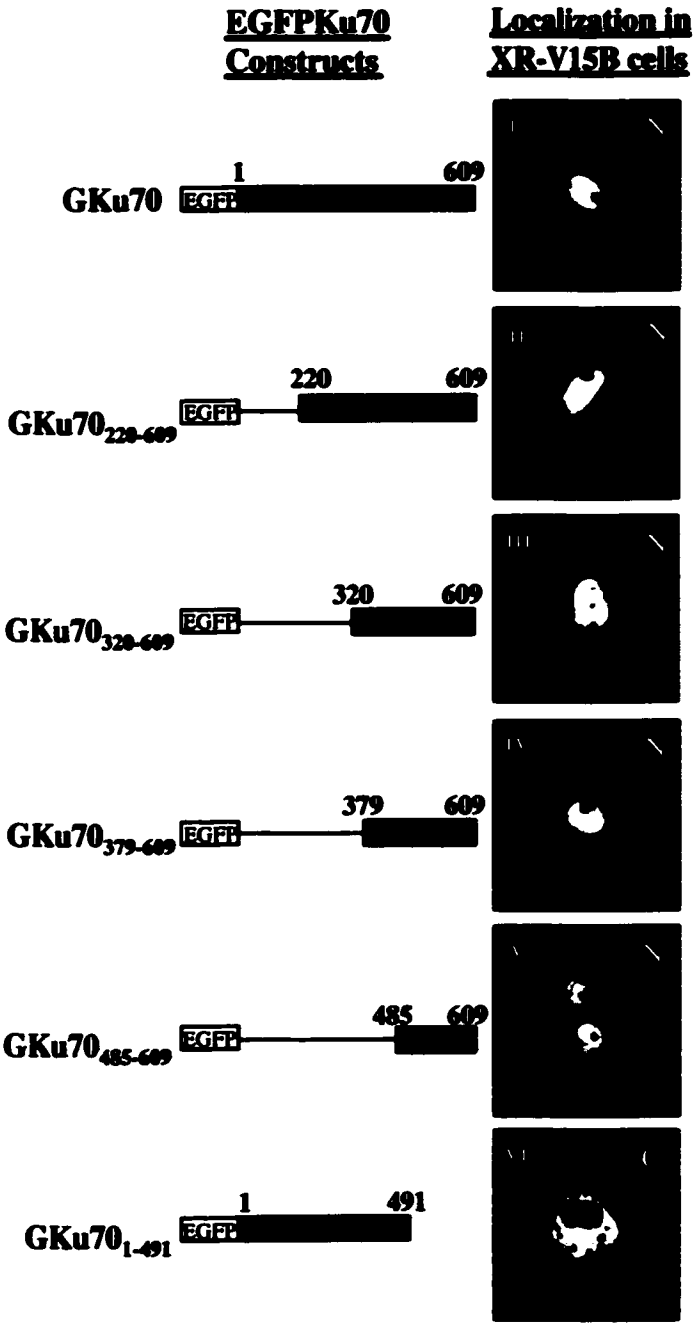
approximately 30 kDa in size that can passively diffuse through the nuclear pore and does not contain subcellular targeting motifs.

Nuclear import of Ku70 is mediated by a basic sequence comprising amino acids 539-556

To identify the signal within Ku70 that mediated its accumulation in the nucleus, I generated a number of EGFP fusion proteins (GKu70) and examined their subcellular localization in XR-V15B cells following expression by transient transfection (Fig. 18). GKu70 N-terminal deletion mutants $GKu70_{220\text{-}609}$, $GKu70_{320\text{-}609}$, $GKu70_{379\text{-}609}$ and $GKu70_{485\text{-}609}$ containing progressively larger deletions of Ku70 to amino acid 485 localized completely to the nucleus (Fig. 18, i-v). The nuclear accumulation of these constructs suggested the nuclear targeting motif was present on all these portions of GKu70 and resided within amino acids 485-609. By contrast, a C-terminal deletion mutant of GKu70, $GKu70_{1\text{-}491}$, missing amino acids 492-609 localized to the cytoplasm (Fig. 18, vi). This result strongly indicated that the NLS has been deleted from this construct.

The most common class of nuclear import signals is referred to as the classical NLSs. These NLSs are composed of short amino acid sequences rich in basic amino acid residues, namely lysines and arginines. These signals can occur as a single cluster or as two smaller clusters of basic residues with a short spacer sequence of 7-10 amino acids. The prototype monopartite and bipartite classical NLSs are those of the SV40 T

Figure 18. Localization of GKu70 deletion mutants in live XR-V15B cells. The localization of a series of recombinant GKu70 constructs expressed by transient transfection and containing N or C-terminal deletions in the Ku70 sequence was determined by direct microscopy of live cells 24 hours post transfection (i-vi). A schematic description of the constructs is shown to the left, while representative photomicrographs of the fluorescent signal observed are shown to the right of each construct. N – Ku70 localized completely to the nucleus, C – Ku70 localized to the cytoplasm. Bar scale, 10 μm.

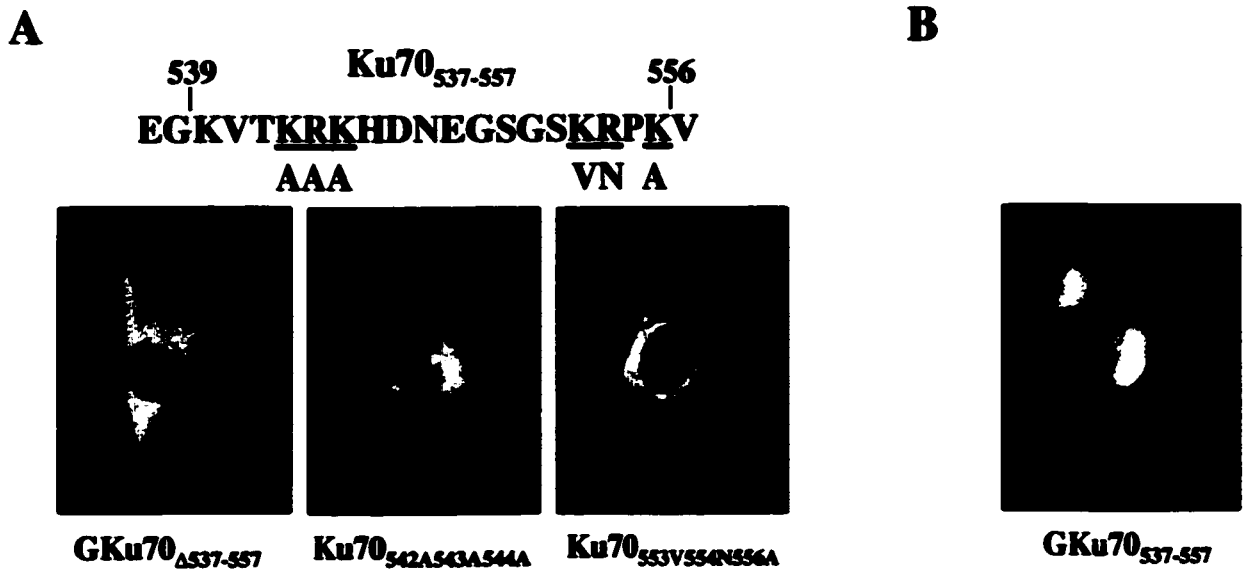

EGFPKu70 Constructs
Localization in XR-V15B cells
GKu70
1
609
EGFP
GKu70220-609
220
609
EGFP
GKu70320-609
320
609
EGFP
GKu70379-609
379
609
EGFP
GKu70485-609
485
609
EGFP
GKu701-491
1
491
EGFP

antigen and nucleoplasmin, respectively (Kalderon et al., 1984; Lanford and Butel, 1984; Robbins et al., 1991).

Inspection of the Ku70 amino acid sequence from 491 to 609 revealed only a single candidate for a basic NLS. This region consisted of two short clusters of basic amino acids between amino acids 539 and 556 separated by an eight amino acid spacer region (Fig. 19A). I found no other candidate motifs in this region of Ku70 and this sequence was conserved in mammalian and avian Ku70 proteins.

To determine whether one or both halves of this potential bipartite basic motif mediated the nuclear localization of Ku70, I examined the localization of three additional Ku70 constructs containing directed mutations in full-length Ku70 (Fig. 19A). GKu70 with a short deletion of 21 amino acids removing the two basic clusters between 537-557 was observed to be predominately cytoplasmic (Fig. 19A, left panel). The cytoplasmic distribution of this construct substantiated the involvement of amino acids 537-557 in the nuclear import of Ku70. I then tested directly the involvement of each basic amino acid cluster within this region for the ability to target Ku70 to the nucleus. I constructed two additional full-length Ku70 constructs containing three amino acid substitutions in one of the basic clusters, amino acids 542-544 or 553-556 (Fig. 19A, middle and right panels) and determined their localization by indirect immunofluorescence with Ku70-specific antibody N3H10. Both $Ku70_{542A543A544A}$ and $Ku70_{553V554N556A}$ were mainly localized to the cytoplasm. This demonstrated that neither basic cluster could function independently as an NLS to promote nuclear localization of Ku70. Together these results demonstrate that Ku70 contains a bipartite basic NLS

Figure 19. Ku70 contains a bipartite basic nuclear import signal. (A) Basic amino acids between 537 and 557 of Ku70 are required for nuclear localization of full-length Ku70 monomers. The top of the figure shows the sequence of Ku70 amino acids 537 to 557 and summarizes the substitutions made in the underlined amino acids in the WT sequence. Below to the left is a direct fluorescent photomicrograph illustrating the localization of GKu70 containing a deletion of the 21 amino acids between 537 and 557 in transiently transfected XR-V15B cells. In the middle is a photomicrograph of indirect immunofluorescence determination of the localization of full-length Ku70 containing alanine substitutions from amino acids 542-544 using antibody N3H10, while to the right is a photomicrograph summarizing the localization of Ku70 containing the substitutions indicated in amino acids 553, 554 and 556. (B) Ku70 amino acids 537-557 are sufficient to direct EGFP to the nucleus. Representative photomicrograph indicating the localization of EGFP fused to a peptide of Ku70 amino acids 537-557. Bar scales, 10 μm.


A
539
Ku70537-557
556
EGKVTKRKHDNEGSGSKRPKV
AAA
VN A
GKu70Δ537-557
Ku70542A543A544A
Ku70553V554N556A
B
GKu70537-557

between amino acids 539 and 556 that is needed for the nuclear localization of Ku70 monomers in XR-V15B cells.

I then tested whether this bipartite motif was also sufficient to mediate nuclear localization of a protein that is normally not actively targeted to the nucleus. I determined that addition of amino acids 537-557 of Ku70 to EGFP was sufficient to promote the complete transfer of EGFP to the nucleus (Fig. 19B). Therefore, the identified sequence that mediates the nuclear import of Ku70 can also function as an NLS in the context of another protein and is not dependent on any other determinants within Ku70 for its activity.

Dimerization of Ku70 and Ku80 subunits *in vivo* is minimally affected by mutations in Ku80 that were previously shown to disrupt heterodimer formation

Endogenous hamster Ku70 is present in XR-V15B cells in very low amounts and its recovery upon expression of Ku80 is slow. Therefore, it was not expected to influence experiments directed towards identifying the motif that mediated the nuclear import of Ku80. Nevertheless, to further minimize the possibility for ambiguity in analysis of Ku80 nuclear uptake in my work, I investigated the possibility of using a mutant form of Ku80 that has previously been shown to block dimerization of *in vitro* translated Ku subunits and of recombinant Ku in yeast-two hybrid experiments. I therefore introduced the two amino acid substitutions described in this study, A453H and V454H, into my EGFPGKu80 (GKu80) construct.

In a first experiment (Fig. 20), I compared the subcellular localization and dimerization of EGFP-containing WT Ku80 and $Ku80_{453H454H}$ in transfected XR-V15B cells using EGFP tags and an antibody, 162, that is specific for human and rodent Ku heterodimers and does not recognize the individual Ku70 or Ku80 monomers. GKu70, GKu80 and $GKu80_{453H454H}$ were each observed by direct fluorescence to be exclusively localized to the nucleus when expressed individually (Fig. 20, ii, iv, vi). As expected, these subunits were not detected by antibody 162 in indirect immunofluorescence experiments (Fig. 20, i, iii, v). This demonstrated that the endogenous hamster Ku70 did not re-accumulate and dimerize with Ku80 to detectable levels during the course of the assay. However, when Ku70 and GKu80 were co-expressed together, antibody 162 readily detected the Ku dimer in the nucleus (Fig. 20, vii). Unexpectedly, expression of Ku70 in combination with $GKu80_{453H454H}$ that contained the amino acid substitutions that were reported to block Ku70/Ku80 dimerization also resulted in prominent nuclear immunofluorescence in response to antibody 162 (Fig. 20, viii). These results indicated that dimerization of Ku in XR-V15B cells was affected only minimally by the A453H and V454H substitutions within GKu80.

To examine the effect of the A453H, V454H substitutions on Ku dimerization in more detail I assessed Ku dimerization in a co-immunoprecipitation assay (Fig. 21). In this experiment, I expressed Ku70 with EGFP-tagged Ku80 or $Ku80_{453H454H}$. Western blot analysis of whole cell extracts revealed that considerably less Ku70 accumulated in the cells in the presence of $GKu80_{453H454H}$ than in the presence of GKu80 (Fig. 21, top

Figure 20. Dimerization of recombinant Ku70 and Ku80 in mammalian cells determined by indirect immunofluorescence. Dimerization of Ku70 and GKu80 was assessed in transiently transfected XR-V15B cells using the dimer-specific Ku monoclonal antibody 162. Cells were transiently transfected to express the Ku70 and GKu80 constructs indicated. The Ku70 constructs have been described in earlier figures. GKu80 represents a full-length Ku80 peptide with EGFP at the N-terminus, while $GKu80_{453H454H}$ is the same peptide as GKu80, except for the inclusion of A453H/V454H substitutions that have been shown previously to abrogate Ku dimerization *in vitro*. Indirect immunofluorescence detection of Ku heterodimers with antibody 162 (i, iii, v, vii, viii) and direct observations of EGFP fluorescence (ii, iv, vi) were made 24 hours post transfection on the same cells by alternating filters to detect either a Red-X conjugated secondary antibody or EGFP following fixation. Bar scale, 10 μm.

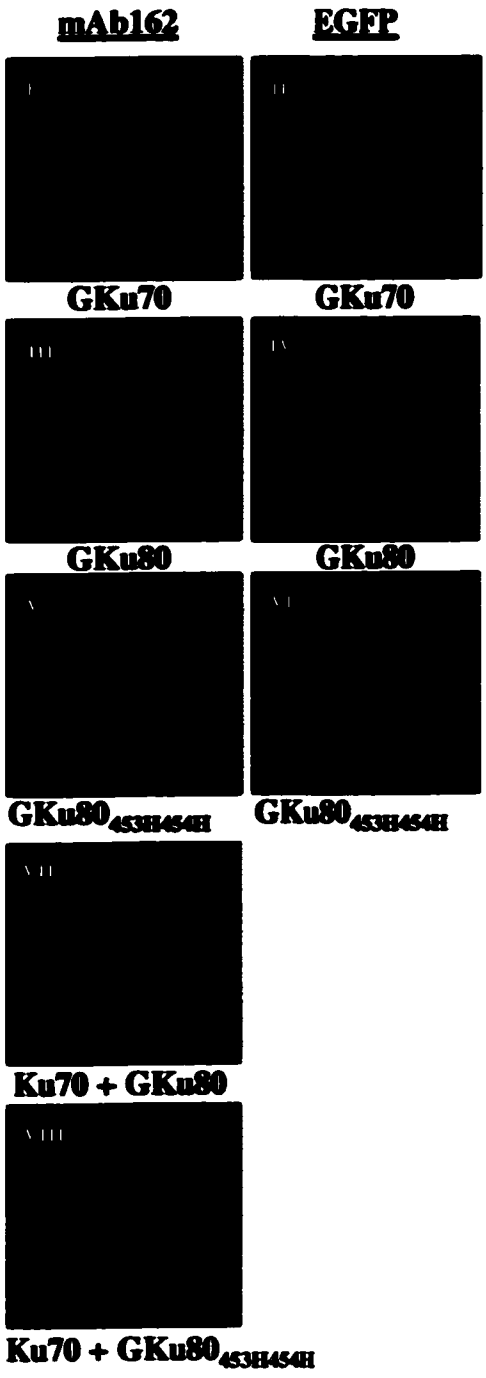
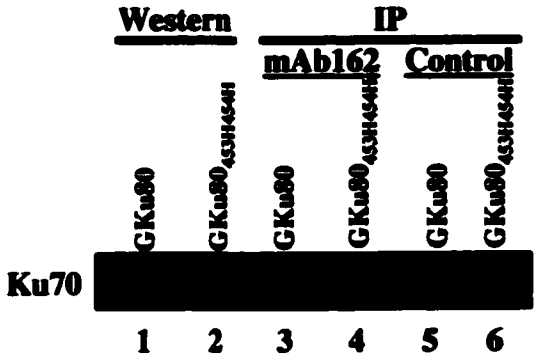
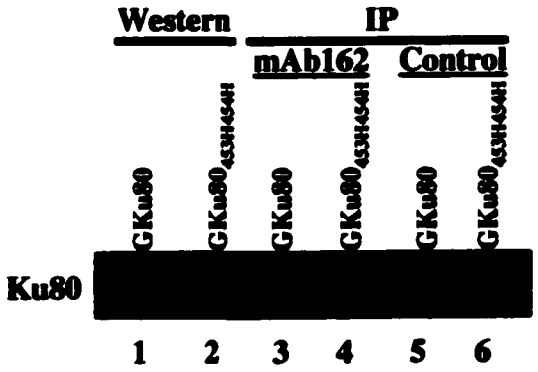

mAb162
EGFP
I
II
GKu70
GKu70
III
IV
GKu80
GKu80
V
VI
GKu80453H454H
GKu80453H454H
VII
Ku70 + GKu80
VIII
Ku70 + GKu80453H454H

Figure 21. Dimerization of Ku subunits determined by co-immunoprecipitation. Western analysis of Ku70 with antibody N3H10 (top) and GKu80 with antibody 111 (bottom). Lanes 1 and 2 show the relative levels of Ku in whole cell extracts prepared from XR-V15B cells transfected to express Ku70 in the presence of GKu80 or $GKu80_{453H454H}$, respectively. 1 μg of cDNA expression construct was transfected for each vector in each experiment, with GKu80 and $GKu80_{453H454H}$ expressed from the same vector. The remaining lanes show a Western analysis of Ku immunoprecipitates from immunoprecipitations performed with the Ku dimer-specific antibody 162 (lanes 3 and 4) or a nonspecific antibody (lanes 5 and 6). All data were reproduced in three independent experiments.


Western
IP
mAb162
Control
GKu80
GKu80453H454H
GKu80
GKu80453H454H
GKu80
GKu80453H454H
Ku70
1
2
3
4
5
6


Western
IP
mAb162
Control
GKu80
GKu80453H454H
GKu80
GKu80453H454H
GKu80
GKu80453H454H
Ku80
1
2
3
4
5
6

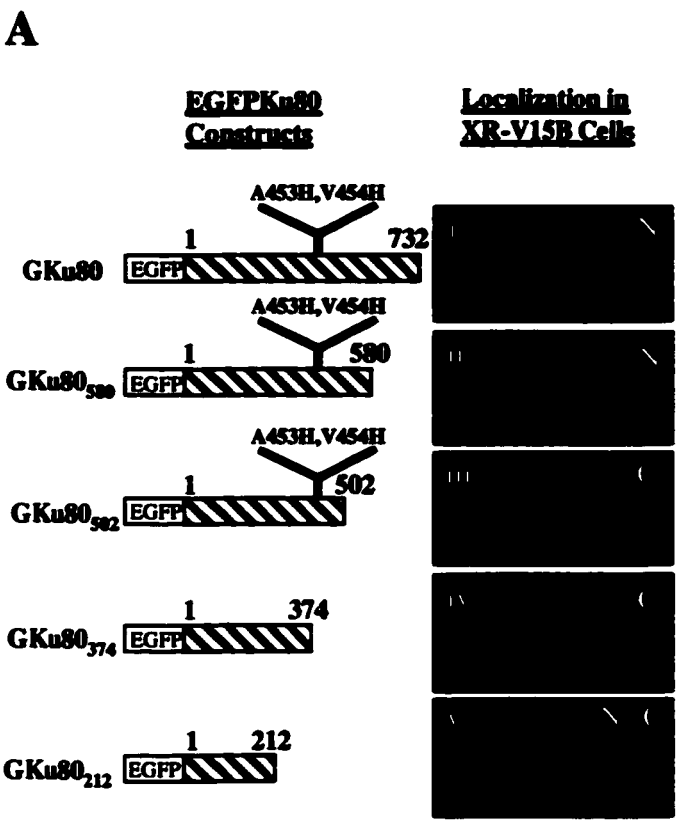
panel, lanes 1 and 2). By contrast, $GKu80_{453H454H}$ accumulated to only a marginally lower level than GKu80 (Fig. 21, lower panel, lanes 1 and 2).

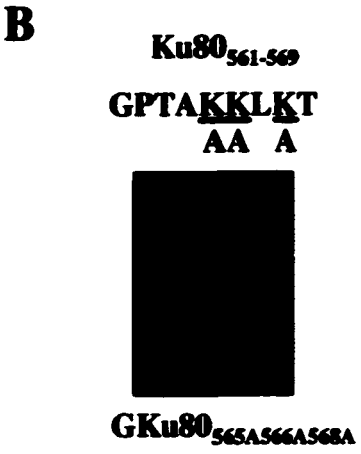
Despite these differences in the level of expression of the Ku subunits, the Ku heterodimer-specific antibody 162 immunoprecipitated Ku heterodimers containing both GKu80 and $GKu80_{453H454H}$ from the cellular extracts (Fig. 21, both panels, lanes 3 and 4). However, a smaller amount of Ku70/$GKu80_{453H454H}$ heterodimer was immunoprecipitated than Ku70/GKu80 dimer (Fig. 21, compare lanes 3 and lanes 4). Nonetheless, the relative amounts of Ku70 co-immunoprecipitated with GKu80 and $GKu80_{453H454H}$ exactly reflected the quantities of Ku70 expressed in the presence of GKu80 and $GKu80_{453H454H}$, respectively (Fig. 21, compare lanes 3 and 4 of the upper panel with lanes 1 and 2). Therefore, my results indicated that the substitutions A453H/V454H in GKu80 selectively promoted a decrease in the steady state levels of Ku70 without affecting the formation of Ku70/GKu80 heterodimers.

Ku80 contains a short basic nuclear import signal encompassing amino acids 565-569

To begin to delimit the nuclear localization sequence within Ku80 I monitored the subcellular distribution of a series of GKu80 fusion constructs containing increasingly larger deletions from the C-terminus (Fig. 22A). Longer constructs contained the $Ku80_{453H454H}$ backbone. Both the full-length GKu80 and a C-terminal deletion mutant lacking amino acids 581-732 localized completely to the nucleus (Fig. 22A, i, ii). Truncation of the C-terminus of GKu80 to amino acid 502 resulted in a GKu80 mutant

Figure 22. Ku80 contains a short basic NLS that is required for nuclear localization of the Ku80 monomer in XR-V15B cells. (A) The localization of a series of recombinant GKu80 constructs expressed by transient transfection and containing C-terminal deletions in the Ku80 sequence was determined by direct microscopy of live cells 24 hours post transfection (i-v). A schematic description of the constructs is shown to the left, while representative photomicrographs of the fluorescent signal observed are shown to the right of each construct. Longer Ku80 constructs contained the A453H/V454H substitution. N – localized completely to the nucleus, C – localized to the cytoplasm, N=C – distributed equally throughout the cell. (B) The amino acid sequence encompassing the basic motif in Ku80 between amino acids 561 and 569 is shown at the top of the panel and illustrates the positions of the substitutions made in $GKu80_{565A566A568A}$. At the bottom of the panel is a representative photomicrograph of EGFP fluorescence illustrating the localization of $GKu80_{565A566A568A}$ in live cells 24 hours post transfection. Bar scales, 10 μm.


A
EGFPKu80 Constructs
Localization in XR-V15B Cells
A453H,V454H
1
732
GKu80
EGFP
A453H,V454H
1
580
GKu80580
EGFP
A453H,V454H
1
502
GKu80502
EGFP
1
374
GKu80374
EGFP
1
212
GKu80212
EGFP


B
Ku80561-569
GPTAKKLKT
AA A
GKu80565A566A568A

that localized to the cytoplasm. This was a strong indication of the presence of a nuclear import signal within this deleted region. Further, as the GKu_{502} construct contains the dimerization interface of Ku80, this result reiterates the lack of a significant interaction of Ku80 with endogenous hamster Ku70, as Ku70/GKu80 heterodimers might be anticipated to be transferred to the nucleus through the Ku70 NLS (Fig. 23B). A larger GKu80 C-terminal deletion construct, $GKu80_{1\text{-}374}$, was also detected in the cytoplasm (Fig. 22A, iv). The $GKu80_{212}$ mutant was distributed equally throughout the cell (Fig. 22A, v). This is an observation that is often made for smaller proteins lacking subcellular targeting signals that are able to gain access to the nucleus by passive diffusion through the nuclear pore. The distribution of $GKu80_{212}$ mimics closely the distribution observed when expressing EGFP alone (see Fig. 17).

To identify the putative NLS between amino acids 502 and 580 I inspected this region of Ku80 for basic elements and initiated a site directed mutagenesis of potential NLSs. In the first instance, I substituted three amino acids in a short basic motif, KKLK, beginning at amino acid 565. Lysine residues were substituted for alanine residues in the full-length GKu80. In contrast to the WT GKu80, $GKu80_{565A566A568A}$ was observed to be almost completely cytoplasmic (Fig. 22B). This data demonstrates that the lysines within amino acids 565 to 568 comprise the core of the nuclear import signal of Ku80.

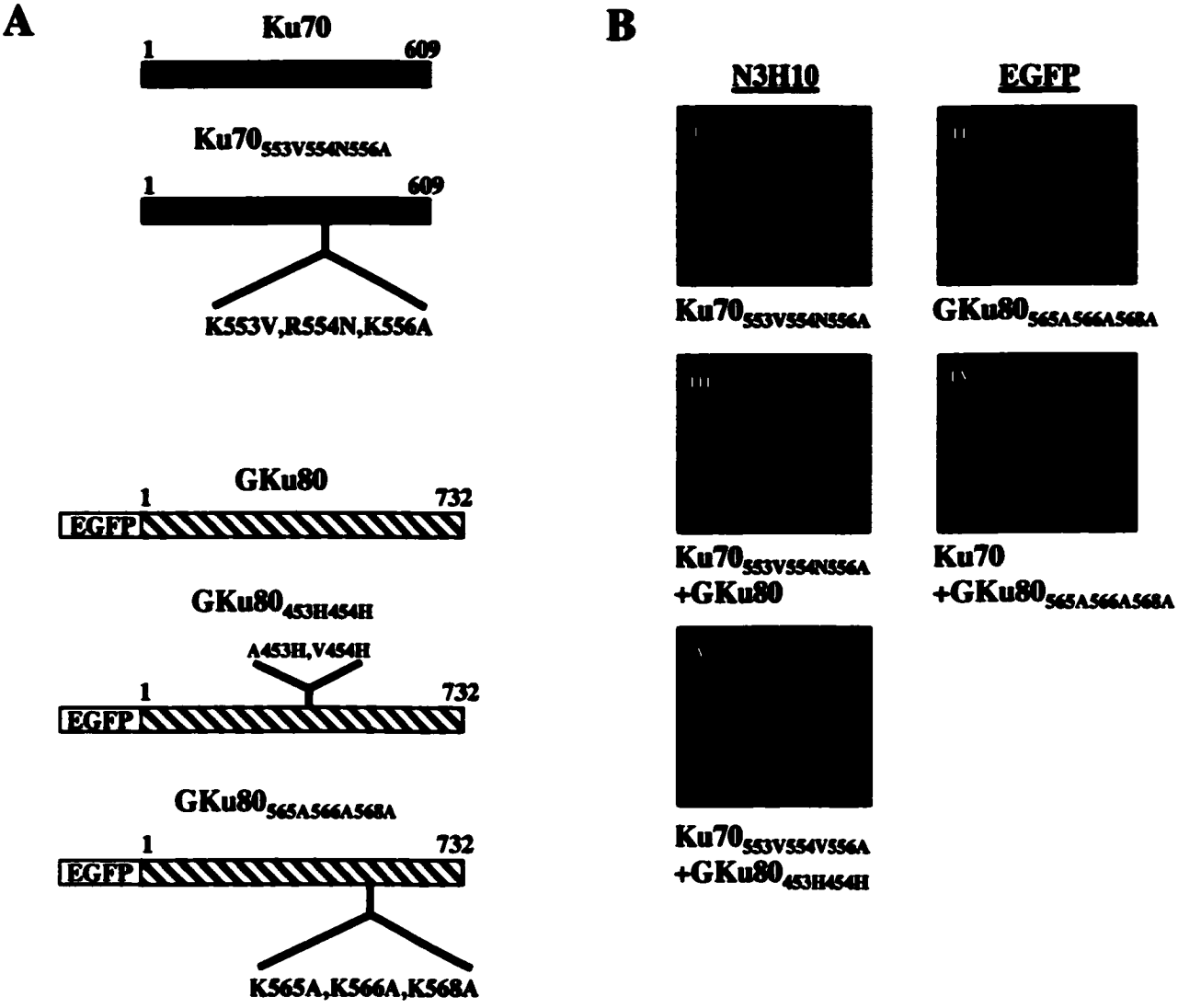
The Ku70 and Ku80 NLSs can independently mediate the nuclear transfer of the Ku heterodimer

Even though I had determined that both Ku70 and Ku80 contained a NLS that was necessary for targeting of the Ku monomers to the nucleus, the extent to which each of these sequences contributed to the nuclear localization of the Ku heterodimer was not known. To answer this question I examined the localization of combinations of nuclear localization competent and nuclear localization deficient Ku70 and GKu80 constructs. The Ku constructs used in the experiments are summarized in Figure 23A.

As expected, expression of $Ku70_{553V554N556A}$ and $GKu80_{565A566A568A}$ in XR-V15B cells resulted in their accumulation in the cytoplasm when expressed alone as they contain inactivating mutations in their NLS (Fig. 23B, i and ii). However, co-expression of each of these mutants with their WT Ku dimerization partner promoted their accumulation in the nucleus (Fig. 23B, iii and iv). These results suggested that each of the Ku NLSs present on the individual Ku monomers was sufficient to promote the nuclear localization of the Ku heterodimer. In addition, $Ku70_{553V554N556A}$ was localized to the nucleus to the same extent when co-expressed with $GKu80_{453H454H}$ (Fig. 23B, v). However, when detecting for $Ku70_{553V554N556A}$ in cells expressing $Ku70_{553V554N556A}$ with GKu80 or $GKu80_{453H454H}$ some cells displayed faint cytoplasmic staining (Fig. 23B, iii and v).

In order to confirm that the accumulation of $Ku70_{553V554N556A}$ and $GKu80_{565A566A568A}$ in the nucleus upon co-expression of the nuclear localization

Figure 23. Nuclear targeting of the Ku heterodimer is accomplished independently by the NLSs of Ku70 and Ku80. (A) Schematic presentation of the Ku70 and GKu80 constructs employed in panel B. (B) Nuclear localization (NL) signal deficient Ku subunits are transported to the nucleus upon co-expression of NL competent dimerization partners. XR-V15B cells were transiently transfected to express the Ku70 and GKu80 constructs as indicated below each photomicrograph (i-v). Localization of Ku70 peptides was determined using indirect immunofluorescence using antibody N3H10, which is specific for Ku70, and a Red-X conjugated secondary antibody (i, iii, v). Localization of GKu80 peptides was determined by direct fluorescence of live cells (ii and iv). Bar scale, 10 μm.


A
Ku70
1
609
Ku70553V554N556A
1
609
K553V,R554N,K556A
GKu80
1
732
EGFP
GKu80453H454H
A453H,V454H
1
732
EGFP
GKu80565A566A568A
1
732
EGFP
K565A,K566A,K568A
B
N3H10
EGFP
Ku70553V554N556A
GKu80565A566A568A
Ku70553V554N556A
+GKu80
Ku70
+GKu80565A566A568A
Ku70553V554V556A
+GKu80453H454H

competent subunits reflected dimerization of Ku subunits, I repeated the analysis of Ku localization with the dimer-specific antibody 162 (Fig. 24). In these experiments, Ku heterodimers, WT (i), GKu80/Ku70$_{553V554N556A}$ (ii), Ku70$_{553V554N556A}$/GKu80$_{453H454H}$ (iii) and Ku70/GKu80$_{565A566A568A}$ (iv) were all observed to be completely localized to the nucleus. These results demonstrated that each of the two NLSs I identified on the individual Ku subunits is sufficient to promote the complete localization of the Ku heterodimer to the nucleus. The data presented in Figure 24 also shows directly that the A453H, V454H substitutions in GKu80 had little effect on dimerization with Ku70 in mammalian cells since Ku70$_{553V554N556A}$ was co-transported to the nucleus with the same efficiency by GKu80 and GKu80$_{453H454H}$. Also, the lack of cytoplasmic staining observed when expressing GKu80/Ku70$_{553V554N556A}$ and Ku70$_{553V554N556A}$/GKu80$_{453H454H}$ when using the Ku heterodimer-specific antibody indicates that the cytoplasmic staining seen when probing for Ku70$_{553V554N556A}$ (Fig. 23B, iii and v) reflects Ku70 monomer.

Finally, co-expression of Ku70 and GKu80 constructs both with inactivating mutations in their NLS, Ku70$_{553V554N556A}$ and GKu80$_{565A566A568A}$, resulted in the formation of a Ku heterodimer that completely localized to the cytoplasm (Fig. 24, v). This result shows that the NLSs that I have identified are the sole NLSs that function to transfer the Ku heterodimer to the nucleus.

Figure 24. Subcellular localization of Ku heterodimers. XR-V15B cells were transiently transfected to express the Ku70 and GKu80 constructs as indicated below each photomicrograph (i-v). Localization of the Ku heterodimer was determined by indirect immunofluorescence using antibody 162 and a Red-X conjugated secondary antibody. Bar scale, 10 μm.

mAb162

I: Ku70 +GKu80

II: $Ku70_{553V554N556A}$ +GKu80

III: $Ku70_{553V554N556A}$ +$GKu80_{453H454H}$

IV: Ku70 +$GKu80_{565A566A568A}$

V: $Ku70_{553V554N556A}$ +$GKu80_{565A566A568A}$

Part II: Discussion

In my analysis of the signals that mediate transfer of Ku to the nucleus I identified two sequences, one present on each of the individual Ku subunits that was required for the nuclear import of the Ku70 and Ku80 monomers. Each sequence can be classified as a classical NLS as the core of both signals was comprised mainly of basic amino acid residues. However, the two NLSs differed considerably in their sequence and composition.

The signal that mediated the transfer of Ku70 to the nucleus was determined to be a bipartite NLS consisting of two clusters of basic amino acid residues separated by an eight amino acid spacer region found between amino acids 539-556. This sequence closely resembled the NLS identified for nucleoplasmin (Robbins et al., 1991). The nuclear import signal of Ku80 was identified as a short motif containing only three basic amino acids starting at amino acid 565. This NLS is similar to the NLS that mediates nuclear import of the SV40 T antigen (Kalderon et al., 1984; Lanford and Butel, 1984).

For the Ku70 NLS I demonstrated that substitution of the basic amino acids at 542, 543 and 544, representing one basic cluster, to alanine residues abrogated the ability of Ku70 to translocate into the nucleus. Point mutations replacing the lysines and arginine residue at amino acids 553, 554 and 556 altering the second basic cluster also resulted in the elimination of Ku70 transfer to the nucleus. These experiments confirmed that each individual basic cluster does not function independently of the other. In addition, the Ku70 NLS was determined to be sufficient to completely transfer EGFP to the nucleus demonstrating that the NLS can function independently of any other

determinants within Ku70. For the Ku80 NLS, site-directed mutations replacing the three lysine residues comprising the core of the NLS with alanine residues disrupted the ability of Ku80 to localize to the nucleus.

Since Ku exists in the cell predominately as a heterodimer of Ku70 and Ku80, I was particularly interested in determining whether the signals I identified for the Ku monomers functioned in the context of the heterodimer. Through expression of a combination of nuclear localization competent and nuclear localization deficient Ku70 and Ku80 subunits I determined that each of the NLSs within the Ku monomers was sufficient to independently localize the Ku heterodimer to the nucleus. Moreover, co-expression of Ku70 and Ku80 subunits each with mutations that abrogate their nuclear transfer resulted in a Ku heterodimer that was localized almost completely to the cytoplasm. This shows that the two NLSs that I have mapped within the Ku70 and Ku80 subunits are the sole NLSs that function to transfer the Ku heterodimer to the nucleus.

Many proteins that are actively imported into the nucleus and contain basic NLSs are imported into the nucleus through the importin α family of proteins that directly recognize the NLS through their armadillo repeats (Conti et al., 1998). Armadillo domains are composed of three alpha helices. A short helix is followed by two longer helices that bundle against each other in an antiparallel fashion. Repeated armadillo domains form a right handed super helix of alpha helices that creates a groove in the superhelix that functions in protein binding.

There have been six importin α proteins identified to date and most are ubiquitously expressed (Kohler et al., 1999). However, at least one shows tissue-specific expression (Kohler et al., 1999). It has been reported that some proteins to be imported

into the nucleus are preferentially imported by a specific member of the importin α family. Also, experiments have shown that when presented with more than one substrate a particular importin α can display preference for import of a specific substrate (Kohler et al., 1999). It is therefore possible that two functional NLSs within the Ku heterodimer increases the efficiency in which Ku is imported into the nucleus in certain cell types by increasing the number of importin α's that can mediate its nuclear import. However, in my experiments with hamster ovarian fibroblast cells inactivation of the NLS in one Ku subunit had little, if any, effect on the efficiency in which the Ku heterodimer was imported into the nucleus.

In my experiments the NLS of either Ku70 or Ku80 was sufficient to completely transfer the Ku heterodimer to the nucleus. So why does Ku contain two NLSs and why are they so different in composition? Since each Ku subunit contains an NLS, it is possible that this may reflect nuclear functions of the individual Ku monomers. However, Ku70 and Ku80 monomers in the absence of their partner are unstable and accumulate only to low levels in the tissues examined to date suggesting that within WT cells Ku monomers are also present in relatively small amounts. Whether these small quantities of Ku70 and Ku80 monomers perform functions within the cell remains to be determined.

Interestingly however, mice engineered to lack Ku70 and Ku80 do display distinct phenotypes. For example, Ku70 deficient mice produce mature T-cells whereas mice lacking Ku80 do not (Gu et al., 1997b; Ouyang et al., 1997). Further, only Ku70 deficient mice develop T-cell lymphoma suggesting a specific role for the Ku70 monomer as a tumour suppressor in T-cells (Gu et al., 1997b; Li et al., 1998).

Alternatively, an NLS on both Ku70 and Ku80 may be required during cell division. A report suggested that Ku is excluded from the nucleus during mitosis and that Ku subunits return to the nucleus as individual monomers with the entry of Ku70 preceeding that of Ku80 in tumour cells (Koike et al., 1999b). A functional NLS present on both subunits may ensure nuclear localization of both Ku70 and Ku80 as cells enter the G_1 phase of the cell cycle. In my experiments however co-expressing mutants of Ku70 and Ku80 compromised for nuclear import resulted in a Ku heterodimer localized to the cytoplasm. In addition, WT Ku subunits were efficient in importing their NLS deficient partner into the nucleus. These results are most consistent with Ku monomers dimerizing in the cytoplasm followed by import into the nucleus of the intact Ku heterodimer.

If the Ku70 and Ku80 subunits enter the nucleus as monomers it would be expected that the nuclear localization deficient Ku70 subunit when expressed with GKu80 would remain in the cytoplasm following cell division. Detecting the localization of a nuclear import deficient Ku70 mutant ($Ku70_{553V554N556A}$) in a large population of transfected cells co-expressing WT GKu80 and $Ku70_{553V554N556A}$ revealed only a fraction of the cells displaying faint cytoplasmic staining. I confirmed that this staining represented the $Ku70_{553V554N556A}$ monomer as no cytoplasmic staining was seen in immunofluorescence experiments when using a Ku heterodimer-specific antibody. Whether this small proportion of $Ku70_{553V554N556A}$ monomer reflects the overexpression of the Ku subunits resulting in a small proportion of the Ku subunits failing to dimerize or whether upon cell division a small population of the Ku heterodimer dissociates

leaving the NLS deficient $Ku70_{553V554N556A}$ monomer in the cytoplasm needs further investigation.

However, if the Ku heterodimer dissociates during cell division it may potentially explain the presence of Ku80 in the cytoplasm of certain cell types. Given the significant differences between the NLSs of Ku70 and Ku80 it is possible that distinct importin α's mediate the nuclear transfer of the two Ku NLSs. In certain tissues or under certain conditions a particular importin α that is required to mediate the import of Ku80 might be limiting. This may result in the distribution of some Ku80 that has not assembled with Ku70 in the cell to the cytoplasm and still allow nuclear accumulation of Ku heterodimer through the Ku70 NLS.

An additional explanation for both Ku subunits containing their own NLS may be a mechanism for the cell to regulate the levels of Ku monomers. Recently, a report has presented evidence that the yeast homologue of importin α (Srp1p) has a distinct function from its well-established role in nuclear import. It was reported that Srp1p functions in the regulation of protein degradation through the ubiquitin-proteosome pathway (Tabb et al., 2000). Therefore, the presence of an NLS on each of the Ku subunits may in some way facilitate their degradation. Signals that mediate nuclear trafficking have already been shown to function in protein turnover for some proteins (Geyer et al., 2000). In this regard it would be interesting to determine whether the half-life of the nuclear localization compromised Ku70 and Ku80 monomers is extended.

The way in which Ku70 and Ku80 monomers dimerize is still poorly understood. Two regions of Ku70 have been identified to interact with Ku80. Both the extreme N-terminus encompassing amino acids 1-115 and a middle portion comprising amino acids

439-482 have been identified as regions that dimerize with Ku80 with the latter representing the major dimerization interface (Jin and Weaver, 1997; Wang et al., 1998a). For Ku80, amino acids 449-477 have been shown to dimerize with Ku70 (Cary et al., 1998; Osipovich et al., 1997; Wang et al., 1998b; Wu and Lieber, 1996). It is apparent that Ku monomers are stabilized by dimerization but whether this is through a dimerization-induced conformational change or by physical masking of degradation signals present on each of the Ku monomers as a result of dimerization is not known. It was proposed for Ku80 that the substitutions A453H and V454H abrogate dimerization with Ku70 based on yeast-two hybrid experiments and experiments with *in vitro* translated Ku (Osipovich et al., 1997). My results suggest that the consequences of these substitutions in Ku80 may be more complex than just disruption of heterodimer formation.

My data show that the Ku heterodimer was immunoprecipitated to the same extent in cells transfected to express Ku70/GKu80 and Ku70/GKu80$_{453H454H}$ when comparing the Ku dimer immunoprecipitated with the amount of Ku70 present in the extract. Also, dimerization of Ku70 with GKu80$_{453H454H}$ was of sufficient stability to allow for co-transport of the NLS deficient Ku70 mutant to the nucleus. However, I did observe that Ku70 accumulated to lower levels when co-expressed with GKu80$_{453H454H}$ than with the WT GKu80. This observation suggests that the mutations in GKu80$_{453H454H}$ reduce the ability of Ku80 to stabilize Ku70 or GKu80$_{453H454H}$ might even directly participate in trans to destabilize Ku70. Whatever the mechanism for the decrease in the levels of Ku70, my result shows that the A453H, V454H mutations within Ku80 in

mammalian cells is more involved than just disruption of heterodimer formation as previously reported.

It has been shown that treatment of CV-1 cells with somatostatin affects the subcellular localization of Ku70 but not Ku80 (Fewell and Kuff, 1996). These experiments suggest that the nuclear translocation of Ku70 and Ku80 may be independently regulated and the subcellular regulation may be partly controlled at the level of Ku70/Ku80 dimerization. Also, Ku70 but not Ku80 was shown to be upregulated upon treatment of cells with ionizing radiation (Brown et al., 2000). This result suggests that under certain conditions Ku70 monomers can accumulate in the cell possibly indicating alterations in the dimerization between the Ku subunits. Given the instability of the individual Ku subunits, one potential mechanism for accumulation of Ku monomers in the cell is by increasing their stability and changing their dimerization capacity. An increase in Ku70 stabilization may partially explain the upregulation in response to ionizing radiation, as mRNA levels were unchanged.

I have noticed as well as others (Chalfie et al., 1994) that addition of the EGFP moiety to Ku80 greatly increases the stability of the Ku80 monomer. My results show that the A453H, V454H mutations allow formation of the Ku heterodimer as well as accumulation of the artificially stabilized GKu80 monomer demonstrating that modifications to a Ku subunit increasing its stability and affecting the Ku70/Ku80 interaction can result in a population of Ku heterodimers and monomers in the cell.

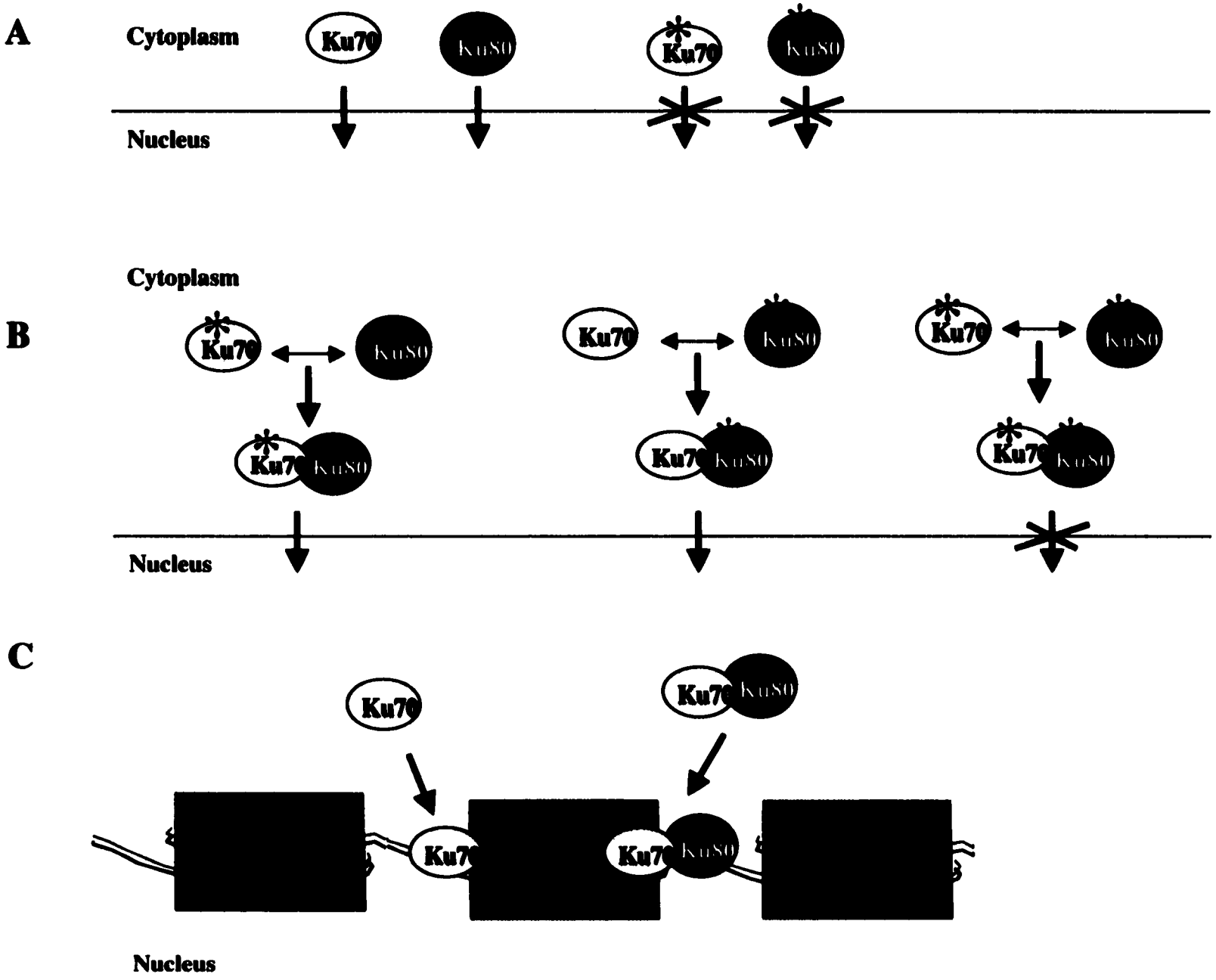
Conclusions

In the work presented I have analyzed the human Ku-dependent activation of reporter genes in yeast and mapped the regions of Ku necessary for this property. Using an HDF2 deficient yeast strain I examined the ability of human Ku to complement specific phenotypes of a yeast strain lacking the Ku80 homologue. I also identified and characterized the signals that mediate the nuclear import of Ku. Further, I presented data on the dimerization and stability of the Ku subunits *in vivo*. A cartoon summarizing the results from my research is presented in Figure 25.

I found that expression of human Ku fused to an ectopic activation domain pleiotrophically activated reporter genes integrated into the yeast genome. The Ku-dependent transcriptional induction was determined to be independent of a UAS, yeast minimal promoter, reporter gene and reporter integration site. Mutational analysis within the amino acid sequence of Ku revealed that the Ku70 monomer was sufficient for activation and both the N and C-terminus were required. Further, internal portions of Ku70 could be removed without abrogating that ability of Ku70 to stimulate transcription of the reporters. By comparing Ku expression in yeast the level of reporter gene activation detected for the Ku heterodimer, Ku70 monomer and Ku70 internal deletion mutants was proportional to the amount of Ku70 protein present in the yeast.

Analysis of the regions of Ku required for reporter gene activation revealed that they shared striking similarities with regions of Ku previously identified to bind to DNA ends *in vitro*. Given that Ku is a prolific DNA binding protein that can bind in a sequence-independent manner to many DNA structures, it seems reasonable to speculate

Figure 25. Pictorial diagram summarizing the experimental results. (A) Wild-type (WT) Ku70 and Ku80 can each enter the nucleus through their own NLS independently of the other Ku subunit. Inactivating mutations in the Ku70 (blue asterisk) or Ku80 (red asterisk) NLS abrogates the ability of the Ku monomers to enter the nucleus. (B) NLS deficient Ku subunits can dimerize in the cytoplasm with their WT partner and translocate to the nucleus. However, a Ku heterodimer lacking the NLS of both Ku subunits remains in the cytoplasm. (C) Once in the nucleus the Ku70 monomer and the Ku heterodimer associate with chromatin in the vicinity of promoters (blue nucleosome).


A
Cytoplasm
Ku70
Ku80
Ku70
Ku80
Nucleus
B
Cytoplasm
Ku70
Ku80
Ku70
Ku80
Ku70
Ku80
Ku70
Ku80
Ku70
Ku80
Nucleus
C
Ku70
Ku70
Ku80
Ku70
Ku70
Ku80
Nucleus

that binding of Ku to DNA mediated the reporter gene activation observed in yeast. Recently, studies examining yeast promoter DNA regions have shown that DNA of promoters adopts a more open structure that is more accessible to DNA binding factors. Therefore, it is possible that yeast promoters adopt a unique DNA/chromatin structure that is in some way attractive to Ku. Furthermore, the finding that the Ku-dependent activation property was not conserved in the yeast homologue indicated that the human and yeast Ku differ in a way that is distinguished by their ability to broadly induce transcription of reporter genes in yeast.

I determined that the Ku heterodimer and Ku70 monomer were tightly associated with the nucleus in mammalian cells. Interestingly, deletion of amino acids 1-57 of Ku70 that disrupted the ability to stimulate transcription of reporters in yeast also resulted in a Ku70 monomer that was less tightly associated with the nucleus in rodent cells. Our lab has now shown that the Ku heterodimer and Ku70 monomer can be released from the nucleus by digesting the genomic DNA with DNaseI. Therefore, the transcriptional induction observed by Ku in yeast may translate to a particular function of Ku requiring an interaction of Ku with chromatin in higher eukaryotes. One possibility is a role for Ku at active chromatin regulating gene transcription. The logical next step would be to use chromatin immunoprecipitation assays to test whether Ku preferentially associates with transcriptionally active chromatin in mammalian cells.

A previous report demonstrated that expression of human Ku70 in a yeast strain lacking HDF1 rescued the TS phenotype of the yeast (Barnes and Rio, 1997). I show in my experiments that expression of human Ku80 does not complement the TS phenotype of a HDF2 deficient strain. These results suggest that at least between yeast and human

Ku it appears that the Ku70 proteins are more functionally conserved. Interestingly however, I found that expression of the Ku heterodimer partially rescued the TS phenotype of the HDF2 deficient strain and this complementation could be compromised by removing amino acids 1-57 of Ku70 in the context of the Ku heterodimer. Moreover, despite a partial rescue of the TS phenotype with expression of the human Ku heterodimer, this same Ku failed to rescue the growth phenotype of HDF2 deficient yeast. These results strongly suggest that the TS and growth phenotypes of yeast lacking HDF2 represent two distinct functions of the yeast Ku and the property of Ku that results in the TS phenotype is more functionally conserved between yeast and human Ku.

Also, I showed that mutations A453H, V454H in Ku80 previously shown to disrupt dimerization of Ku70 with Ku80 minimally affected heterodimer formation *in vivo* but significantly decreased the levels of Ku70 in the cell. These data suggest that dimerization of Ku70 with the mutant Ku80 does not stabilize Ku70. This may result from an altered conformation of the Ku heterodimer that may not mask putative degradation signals of Ku70. Alternatively, the Ku80 mutant through dimerization with Ku70 may actively participate in facilitating the degradation of Ku70. Nevertheless, my experiments show that dimerization of the Ku subunits *in vivo* is more elaborate than indicated by biochemical studies.

Finally, in the experiments designed to characterize the signals that mediate the nuclear transfer of Ku, I determined that both Ku70 and Ku80 contain their own NLS that is sufficient to promote complete nuclear accumulation of the Ku monomers. Each of the import sequences identified within the individual Ku subunits were also efficient in transferring the Ku heterodimer into the nucleus demonstrating that both these signals

function in the context of the Ku heterodimer. The efficient import of the NLS deficient Ku subunit by its WT partner showed that the Ku70 and Ku80 subunits dimerize in the cytoplasm followed by the nuclear import of the intact Ku heterodimer. Point mutations within each of the NLSs resulted in a Ku heterodimer that was predominately localized to the cytoplasm which indicates that the two NLSs that I have mapped are the sole signals that mediate nuclear uptake of Ku.

Characterization of the NLSs of Ku70 and Ku80 revealed that they differ considerably in their composition. Although both import sequences can be classified as classical NLSs, as their core is comprised mainly of basic amino acids, the Ku70 NLS contains two basic amino acid clusters separated by an eight amino acid spacer while the Ku80 NLS is a short sequence with the core made up of just three basic amino acids. The presence of a NLS on both Ku subunits that are significantly different may indicate a role for the NLSs in regulating the function of the individual Ku subunits under certain conditions or in specific tissues. The activation of yeast reporters by the Ku70 monomer is also consistent with a possible biological role for Ku monomers in the cell. Given the differences in the NLSs of the Ku subunits, it would be of interest to examine the efficiency of import of Ku70 and Ku80 by specific isoforms of importin α. These experiments may provide important information regarding the mechanisms determining the broad subcellular distribution reported for Ku in different tissues.

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APPENDICES

APPENDIX A

Interaction of Ku with TBP is mediated by DNA

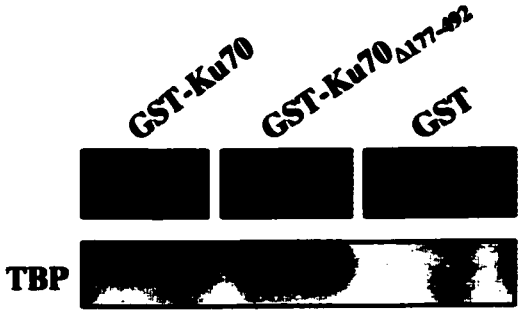
TBP is a ubiquitous protein that functions in RNA polymerases I, II and III transcription. A previous report had shown that Ku bound to the 'CTC box' of the collagen IV genes formed a complex with TBP. I had determined that Ku expressed fused to an ectopic activation domain in yeast activated reporter genes integrated into the yeast genome. To investigate whether Ku and TBP function together in the cell to regulate gene transcription, I first began by examining in more detail the Ku/TBP interaction.

Bacterially purified GST fusions of Ku70 and an internal deletion mutant of Ku70 lacking amino acids 177-492, GSTKu70$_{\Delta 177\text{-}492}$, interacted with TBP from a HeLa whole cell extract (WCE). Figure 26A shows that GSTKu70 and GSTKu70$_{\Delta 177\text{-}492}$ precipitated TBP in a GST pull-down experiment. The presence of TBP was dependent on Ku70 as no TBP was detected when the extract was incubated with GST alone. Figure 26A (top) shows the relative amounts of the GST proteins incubated with the extract. Further, bacterially purified 6xHisKu70 interacted with purified GST-TBP as GST-TBP bound to beads precipitated 6xHisKu70 (Figure 26B).

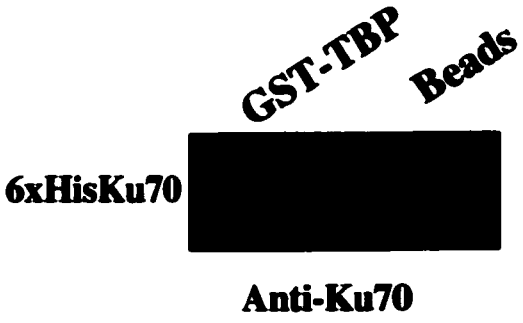
To analyze the Ku/TBP interaction in a more natural situation I immunoprecipitated Ku from a HeLa WCE and examined the immunoprecipitate for the presence of TBP. TBP from a HeLa WCE is immunoprecipitated with Ku-specific antibody 162 but not with a nonspecific MEK-1 antibody or protein A beads alone

Figure 26. Ku70 interacts with TBP *in vitro*. (A) TBP interacts with Ku70 and a internal deletion mutant, $Ku70_{\Delta 177\text{-}492}$. Full-length GST-Ku70, GST-$Ku70_{\Delta 177\text{-}492}$ or GST alone bound to beads was incubated 8 h at 4 °C with 1 mg of HeLa whole cell extract (WCE). Beads were washed 5 times with binding buffer and then heated at 95 °C for 5 min in the presence of 2XSDS sample buffer. The beads were centrifuged and the supernatants were run on a 10 % SDS-PAGE gel. Western blot analysis was performed and TBP detected with a TBP-specific antibody, SI-1 (bottom). Coomassie staining of a SDS-PAGE gel showing relative amounts of the GST fusion proteins used in the pull-down assay (top). (B) Purified 6xHisKu70 interacts with GST-TBP. 6xHis-Ku70 produced from bacteria was incubated for 4 h at 4 °C with GST-TBP bound to beads (GST-TBP) or beads alone (Beads) as indicated. The beads were washed 5 times with binding buffer and then heated at 95 °C in the presence of 2XSDS sample buffer. The samples were centrifuged and the supernatants were run on a 10 % SDS-PAGE gel. Bound 6xHisKu70 was detected by Western blot analysis using the Ku70-specific antibody, N3H10.

A


GST Pull-Down (HeLa WCE)
GST-Ku70
GST-Ku70Δ177-492
GST
TBP

B


GST Pull-Down
GST-TBP
Beads
6xHisKu70
Anti-Ku70

(Figure 27). Also, I analyzed the immunoprecipitate for specific components of known TBP-containing complexes. I found that TAFIIp250 and TAFIIp130 that are components of the TFIID complex and required for RNA polymerase II transcription are not immunoprecipitated with Ku (Fig. 28). Further, a component of the SL1 complex required for RNA polymerase I transcription, TAFIp95/110, was not detected in the immunoprecipitate containing Ku (Fig. 28). However, TBP and DNA-PKcs were both efficiently immunoprecipitated with Ku (Fig.28).

Interestingly, TBP was not detected in a immunoprecipitate containing Ku from HeLa nuclear extracts (Fig. 29A). Also, the addition of ethidium bromide, increasing concentration of highly sheared salmon sperm DNA and DNaseI to the HeLa WCE prior to immunoprecipitation with Ku-specific antibody 162 abrogated the Ku/TBP interaction reflected by less TBP detected in the precipitate (Fig. 29 B, C, D). However, the interaction of Ku with TBP was specific for TBP, as another DNA binding transcription factor, Sp1, is not immunoprecipitated with Ku from a HeLa WCE (Fig. 30).

Together, these results indicate that in the context of a cellular extract DNA facilitates the Ku/TBP interaction. The absence of TAFIIp250, TAFIIp130 and TAFIp95/110 in the Ku/TBP immunoprecipitate would suggest that the Ku/TBP interaction is independent of the TFIID and SL1 complexes. Whether Ku interacts with free TBP or TBP bound to other proteins in addition to those tested for in my experiments remains to be determined. Moreover, since DNA-PKcs was also detected in the Ku immunoprecipitate containing TBP, it is possible that TBP may interact preferentially with Ku when associated with DNA-PKcs. It has already been demonstrated that TBP can be phosphorylated by DNA-PK *in vitro*.

Figure 27. TBP immunoprecipitates with Ku from a HeLa whole cell extract. (A) HeLa WCEs (500 μg of total protein) were incubated with Ku-specific antibody 162 (162), a nonspecific MEK-1 antibody (MEK-1) or no antibody (Beads) for 6 h at 4 °C with gentle shaking. Protein A sepharose beads were then added to all three samples for 1 hour at 4 °C. The beads were washed 5 times with immunoprecipitation (IP) buffer and immunoprecipitates were run on a 10 % SDS-PAGE gel and proteins detected by Western blot. WCE representing 25 % of the input in each of the IP reactions was included to verify the efficiency of the immunoprecipitation as indicated. Ku70 and Ku80 were detected with Ku70 and Ku80-specific antibodies N3H10 and 111, respectively. TBP was detected with antibody SI-1.

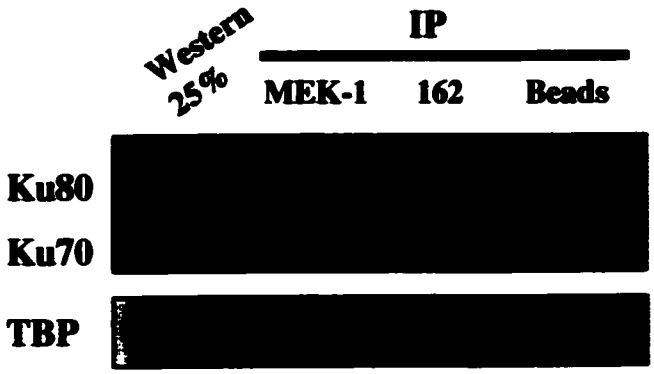

Western 25%
IP
MEK-1
162
Beads
Ku80
Ku70
TBP

Figure 28. TBP and DNA-PKcs but not TBP associated factors are immunoprecipitated with Ku from a HeLa WCE. 500 μg of HeLa WCE was incubated for 2 h at 4 °C with antibody 162 (162) or a nonspecific MEK-1 (MEK-1) antibody. Protein A sepharose beads were added for 1 h. Beads were washed 5 times with IP buffer and an equal amount of the immunoprecipitate was run on a 10 % SDS-PAGE gel for Western blot analysis. Included on the gel is 20 % of the extract used in each IP reaction to verify IP efficiency. The membrane was sequentially probed with antibodies specific for DNA-PKcs (Ab-4), TAFIIp250 (6B3), TAFIIp130 (4A6), TAFIp95/110 (C-18), Ku80 (111), Ku70 (N3H10) and TBP (SI-1).

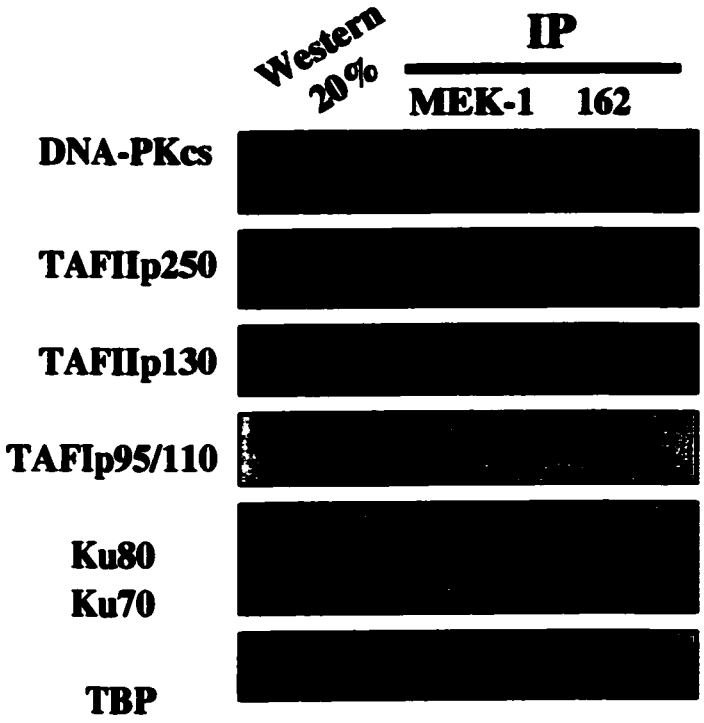
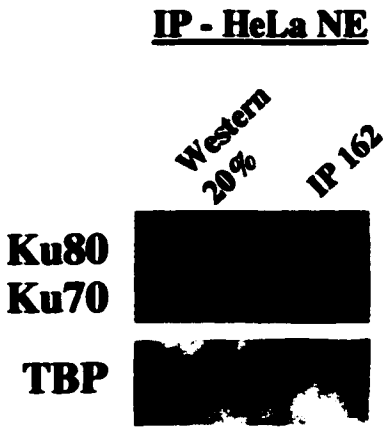
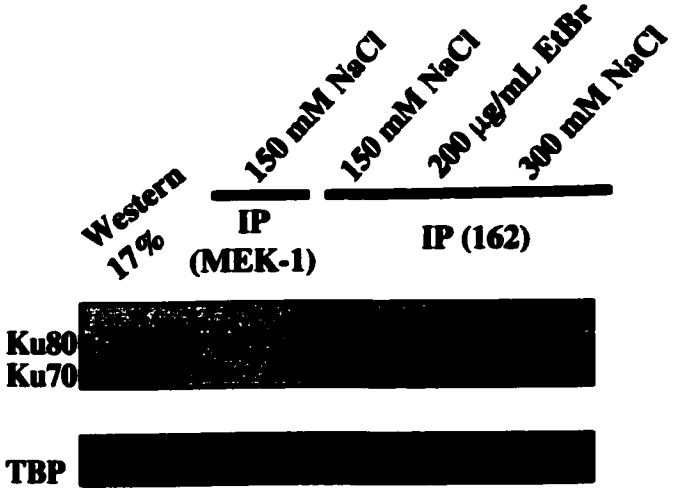

Western 20%
IP
MEK-1
162
DNA-PKcs
TAFIIp250
TAFIIp130
TAFIp95/110
Ku80
Ku70
TBP

Figure 29. Interaction of Ku with TBP from a HeLa WCE is mediated by DNA. (A) TBP does not immunoprecipitate with Ku from a HeLa nuclear extract (NE). 300 μg of NE from HeLa cells was incubated with Ku-specific antibody 162 (IP 162) for 4 h at 4°C with gentle shaking. Protein A sepharose beads were added for 1 h and then the beads were washed 5 times with IP buffer. The immunoprecipitate was run on a 10 % SDS-PAGE gel for Western analysis. 20 % of the extract used in the IP reaction was included on the gel to determine IP efficiency as indicated. The membrane was sequentially probed with antibodies specific for TBP (SI-1), Ku70 (N3H10) and Ku80 (111). (B) The Ku/TBP interaction is abrogated by the addition of ethidium bromide (EtBr) to the extract. Western blot of immunoprecipitates from a HeLa WCE using antibody 162. HeLa WCEs (200 μg per reaction) were incubated with a nonspecific MEK-1 antibody (MEK-1) or antibody 162 (162) to immunoprecipitate Ku in the presence of 150 mM NaCl (final), 300 mM NaCl (final), or 200 μg/ml (final) of EtBr. Co-IP of TBP with Ku is minimally affected by 300 mM NaCl but is significantly reduced by the presence of EtBr. (C) Addition of increasing amounts of highly sheared salmon sperm DNA (SSDNA) proportionally reduces the amount of TBP immunoprecipitated with Ku. Western blot of Ku and TBP immunoprecipitated from HeLa WCEs with antibody 162. 200 μg of HeLa WCE was incubated with antibody 162 or a MEK-1 antibody as indicated with increasing amounts (0.05 μg/μl and 0.45 μg/μl) of highly sheared SSDNA as indicated at the top. As the amount of SSDNA increases the relative amount of TBP immunoprecipitated with Ku decreases proportionally while the amount of Ku immunoprecipitated from the extract is unaffected. (D) DNaseI treatment of the HeLa WCE abolishes the Ku/TBP interaction. WCEs from HeLa cells were incubated with antibody 162 in the presence or absence of DNaseI or with the MEK-1 antibody as indicated. No TBP is immunoprecipitated from the extract that was incubated with DNaseI while the amount of Ku immunoprecipitated was unaffected.

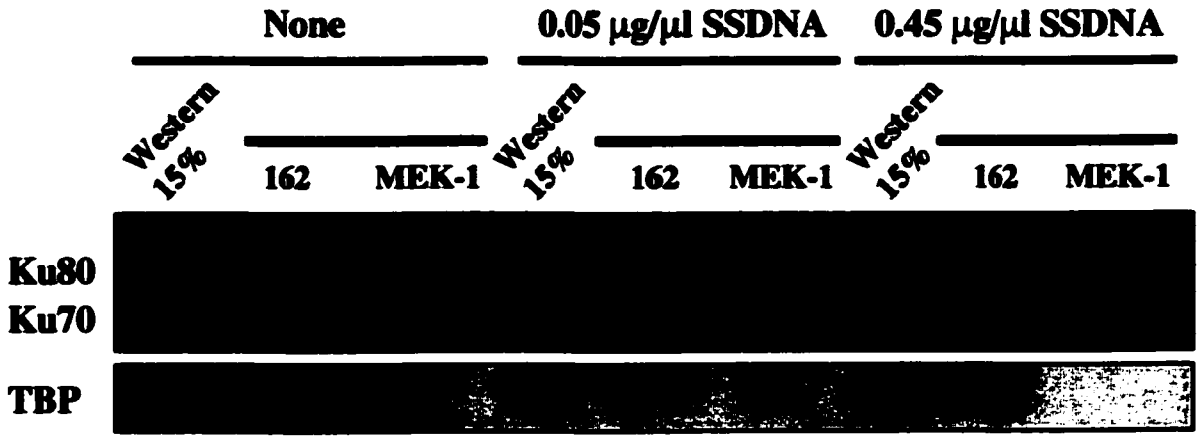
A


IP - HeLa NE
Western 20%
IP 162
Ku80
Ku70
TBP

B


Western 17%
150 mM NaCl
150 mM NaCl
200 µg/mL EtBr
300 mM NaCl
IP (MEK-1)
IP (162)
Ku80
Ku70
TBP

C


None
0.05 µg/µl SSDNA
0.45 µg/µl SSDNA
Western 15%
162
MEK-1
Western 15%
162
MEK-1
Western 15%
162
MEK-1
Ku80
Ku70
TBP

D

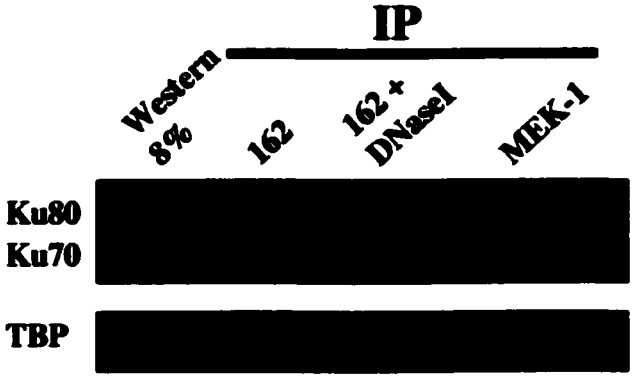
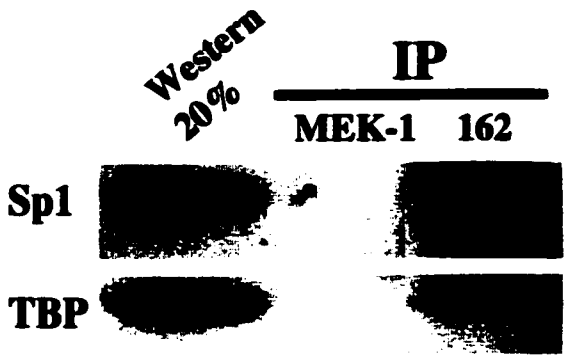

IP
Western 8%
162
162 + DNaseI
MEK-1
Ku80
Ku70
TBP

Figure 30. TBP but not Sp1 immunoprecipitates with Ku from a HeLa WCE. HeLa WCEs (500 μg) were incubated with Ku-specific antibody 162 (162) or a MEK-1 nonspecific antibody (MEK-1) for 2 h at 4 °C as shown. Protein A sepharose beads were added for 1 h at 4 °C and the beads were then washed 5 times with IP buffer. Immunoprecipitated proteins were analyzed by Western blot with TBP and Sp1-specific antibodies SI-1 and 1C6, respectively. Included on the gel is 20 % of the extract used in the IP reactions to estimate IP efficiency. Only TBP is detected in the immunoprecipitate using antibody 162.


Western
20%
IP
MEK-1
162
Sp1
TBP

APPENDIX B

Export of Ku70 and Ku80 from the nucleus likely occurs through the Crm1-mediated pathway

To begin to study the nuclear export of Ku I (in collaboration with Rhian Walther) employed a variation of the FRAP (fluorescence recovery after photobleaching) technique that was developed in our laboratory. Our laboratory has established that this system accurately reflects the nuclear export of proteins using control peptides (Walther et. al. manuscript in preparation). Essentially the protein of interest containing a NLS, that will target the protein to the nucleus, is transiently expressed in Cos7 cells as an EGFP fusion. By direct visualization of EGFP fluorescence, a live, multinucleated cell expressing the protein is selected and the EGFP signal from one nucleus irreversibly photobleached with a laser. The recovery of the EGFP signal is then monitored over a period of time. Recovery of the signal reflects export of the protein from the non-bleached nucleus and accumulation into the bleached nucleus. In all assays cycloheximide was added to eliminate *de novo* protein synthesis over the time course of the experiment.

The EGFPKu70 (GKu70) and EGFPKu80 (GKu80) constructs that I had previously generated to study the nuclear import of Ku were tested for their ability to be exported from the nucleus. Full-length GKu70 and GKu80 were transiently expressed in Cos7 cells. Since I determined previously that both Ku70 and Ku80 contain a natural

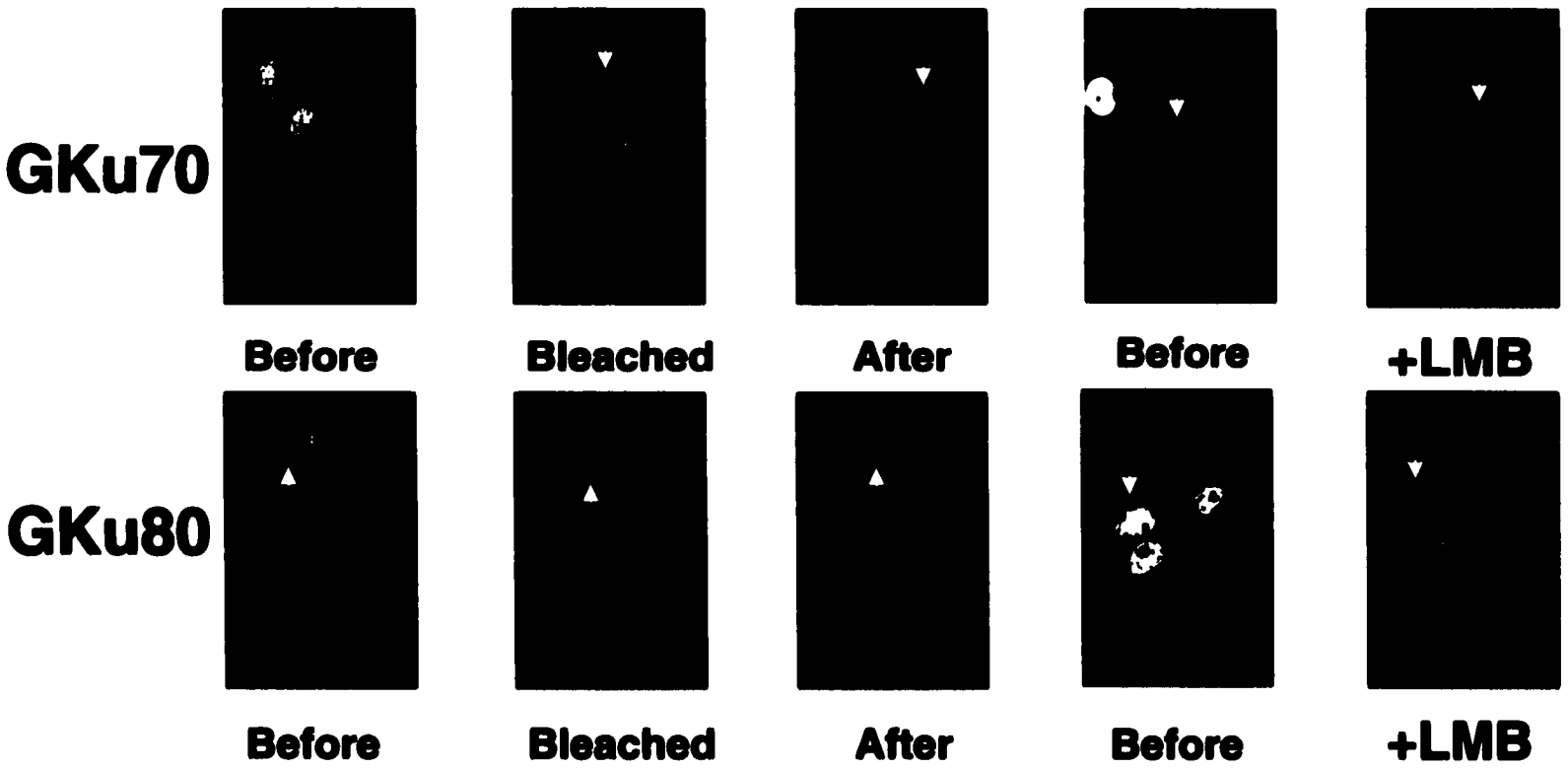
NLS and completely accumulated into the nucleus of XR-V15B cells these same constructs were also expected to completely localize to the nucleus in Cos7 cells.

Figure 31, before panel shows that both GKu70 and GKu80 completely transfer to the nucleus in Cos7 cells. A signal nucleus of a multinucleated cell was then irreversibly bleached (white arrows, bleached panel) and the recovery of the signal monitored for 15 minutes. Almost complete recovery of the signal was seen with both GKu70 and GKu80 after 15 minutes (after). Upon treatment of the cells with LMB and inhibitor of Crm1-mediated nuclear export, the amount of recovery in 15 minutes was dramatically reduced (+LMB, white arrows). Also, for GKu70 treated with LMB, GKu80 and GKu80 treated with LMB the nucleus of a singly nucleated cell was bleached (red arrows) to control for nuclear export-independent recovery of the EGFP signal. These preliminary experiments were repeated approximately three times and 6-8 cells in total were analyzed for each condition. Variations in the times required for recovery of the signals were noticed between experiments. However, this preliminary data indicates that Ku70 and Ku80 are proteins that continuously shuttle between the nucleus and cytoplasm and are exported from the nucleus by the Crm1-mediated pathway.

Inhibition of the 26S proteosome and nuclear export may independently stabilize Ku80

I also began to examine whether nuclear export played a role in the turnover of Ku. The degradation of many cellular proteins is through the ubiquitin/proteosome pathway. The 26S proteosome is a dynamic mutisubunit complex that mediates the

Figure 31. Ku70 and Ku80 are exported from the nucleus by the Crm1-mediated pathway. A schematic representation of the EGFPKu70 and EGFPKu80 constructs used to measure nuclear export of Ku is presented at the bottom. A single nuclei of a multinucleated Cos7 cell transiently transfected to express EGFP tagged Ku70 or Ku80 (GKu70 and GKu80, respectively) was irreversibly bleached to ablate EGFP fluorescence (Before, white arrows). Recovery of fluorescence was monitored over a period of 15 minutes. Almost complete recovery of the signal was observed after 15 minutes (After) indicating nucleo-cytoplasmic shuttling of the Ku proteins. No significant recovery of EGFP signal was observed in the presence of leptomycine B (10 nM final) after 15 minutes (+LMB, white arrows) demonstrating that the nuclear export of Ku is through the Crm1-mediated pathway. In GKu70 treated with LMB, GKu80 and GKu80 treated with LMB a nuclei of a singly nucleated cell near the multinucleated cell was bleached (red arrows) to control for nuclear export-independent recovery of EGFP fluorescence.


GKu70
Before
Bleached
After
Before
+LMB
GKu80
Before
Bleached
After
Before
+LMB


GKu70
EGFP
GKu80
EGFP

degradation of cellular proteins following attachment of a multi-ubiquitin chain that is thought to improve targeting to the proteosome.

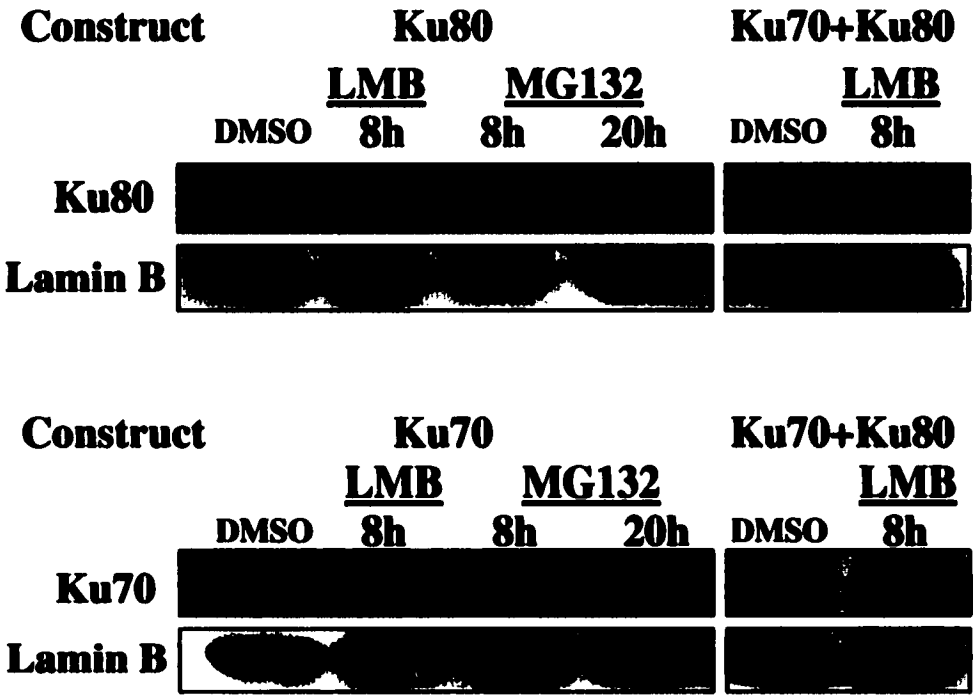
The Ku heterodimer is a stable protein, however the individual Ku subunits in the absence of their partner are unstable reflected by dramatically reduced levels of Ku70 and Ku80 in Ku80 and Ku70 deficient mammalian cell lines, respectively. To begin to study whether the nuclear export of Ku was required for its degradation, I transfected XR-V15B cells to express Ku70, Ku80 or both Ku70 and Ku80. The cells were then treated with MG132, LMB or DMSO for the indicated time periods 24 h post transfection (Fig. 32A). MG132 is an inhibitor of the 26S proteosome that has been widely used in studying protein degradation mediated through the proteosome.

Western analysis shows that treatment of cells with both MG132 and LMB selectively stabilizes Ku80 when expressed alone or in combination with Ku70 as seen by the increase in the level of Ku80 in the cells when compared to cells treated with DMSO (Fig. 32A, top). MG132 and LMB have no detectable affect on the stability of Ku70 (Fig. 32A, bottom). In each of the Western blots the membrane was stripped a reprobed for lamin B to control for protein loading (Fig. 32A).

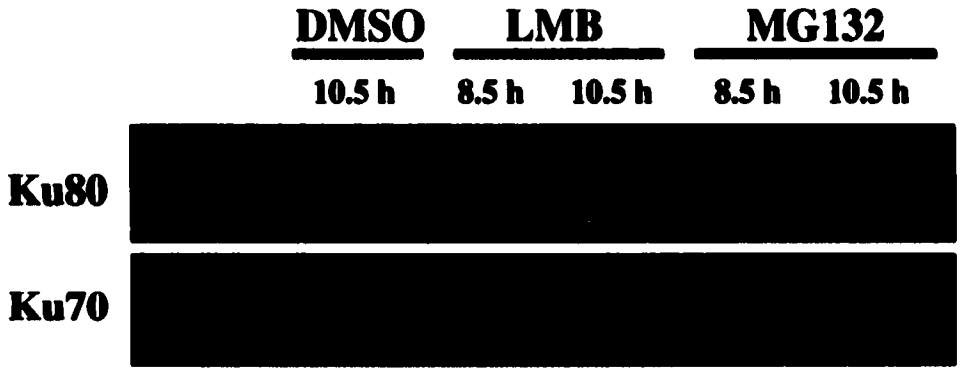
Figure 32B shows Western blots of endogenous Ku from Cos7 cells following treatment with MG132, LMB or DMSO as indicated. There is no detectable increase in the level of Ku70 or Ku80 after MG132 or LMB treatment of cells compared to treatment with DMSO (Fig. 32B). These results most likely reflect the slower turnover of the Ku heterodimer.

Figure 32. Inhibition of the 26S proteosome (MG132) and nuclear export (LMB) independently stabilize Ku80. (A) Treatment of cells with MG132 and leptomycine B (LMB) stabilize Ku80. Western blots of whole cell extracts from Ku deficient XR-V15B cells transfected to express Ku70, Ku80, or both Ku70 and Ku80 as indicated at the top. 24 hours post transfection cells were treated with LMB (10 nM final), MG132 (2 μM final) or with DMSO for the indicated time period. Extracts (100μg per lane) were run on a 10 % SDS-PAGE gel, transferred onto membrane and probed with either a Ku70 (N3H10) or Ku80 (111)-specific antibody. Membranes were stripped and reprobed with a lamin B antibody to control for protein loading. (B) Western blots of whole cell extracts from Cos7 cells treated with LMB (10 nM final), MG132 (2 μM final) or DMSO for the time periods indicated. Extracts (50 μg per lane) were subjected to Western analysis and endogenous Ku70 and Ku80 detected with antibodies N3H10 and 111, respectively.

A


Construct
Ku80
Ku70+Ku80
LMB
MG132
LMB
DMSO
8h
8h
20h
DMSO
8h
Ku80
Lamin B
Construct
Ku70
Ku70+Ku80
LMB
MG132
LMB
DMSO
8h
8h
20h
DMSO
8h
Ku70
Lamin B

B


DMSO
LMB
MG132
10.5 h
8.5 h
10.5 h
8.5 h
10.5 h
Ku80
Ku70

Together, this data suggests that the degradation of Ku80 is at least facilitated by its nuclear export and is at least partially through the 26S proteosome. Also, the results suggest that either the degradation of Ku70 is not dependent on its nuclear export and is degraded by a different pathway than Ku80 or the turnover of Ku70 is considerably slower than that of Ku80 and therefore an increase in the level of Ku70 is not detected over the time periods of the treatments. The selective increase in Ku80 upon the transient co-expression of both Ku70 and Ku80 (Fig. 32A, top) is most likely a result of an excess of Ku80 monomers in the cell that have not dimerized with Ku70, probably do to overexpression of the individual Ku subunits. These Ku80 monomers would be expected to turnover as rapidly as Ku80 monomers when expressed in the absence of Ku70.

APPENDIX C

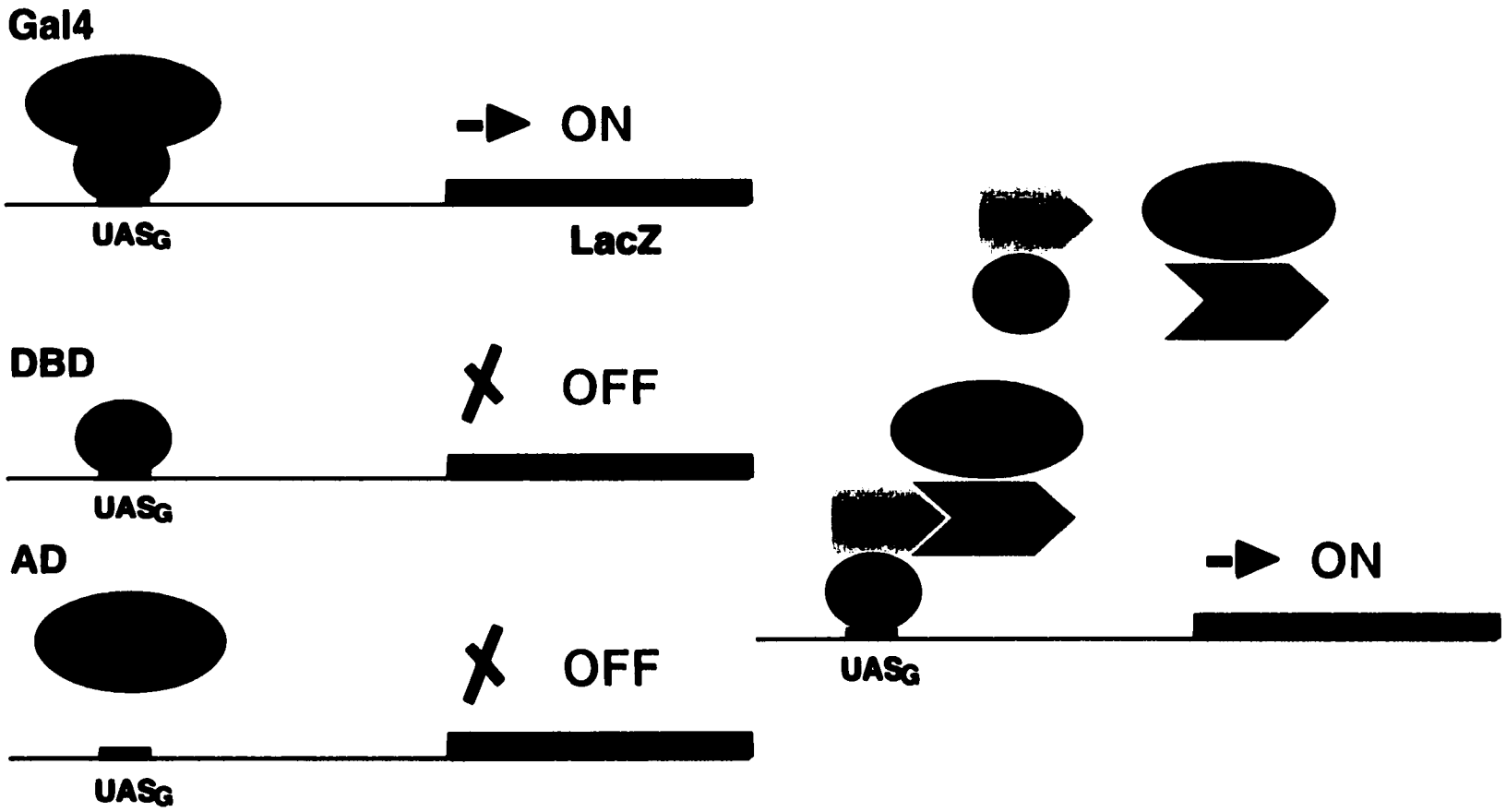
Yeast two-hybrid screening for putative Ku80 binding proteins

Since Ku deficient mice and cell lines display distinct phenotypes from mice or cell lines lacking DNA-PKcs it is likely that Ku interacts functionally in the cell with other proteins in cellular processes that are independent of Ku's role as a regulator of DNA-PKcs activity. Therefore, my objective was to use the yeast two-hybrid technique to identify putative Ku80 binding proteins. Characterization of novel proteins that interact with Ku would provide important information on the functions of Ku in the cell. A schematic illustration of the principle behind the yeast two-hybrid technique is presented in Figure 33.

A Jurkat T-cell cDNA library was chosen to screen for putative Ku80 binding proteins. A Jurkat library was chosen as Ku was reported to be enriched in T-cells and is known to play a crucial role in V(D)J recombination. Therefore, it was possible that this library may have contained specific Ku binding proteins that are not present in libraries derived from other tissues.

Prior to my arrival another member of the laboratory had begun screening the library with the full-length Ku80. It was found that the Ku80 expressing plasmid was spontaneously recombining in the yeast impeding the library screening. Therefore, I chose to screen the library with portions of Ku80 in the hope that the spontaneous recombination of a plasmid with smaller segments of the Ku80 cDNA would not occur.

Figure 33. Schematic illustration of the yeast two-hybrid system used to screen for putative Ku80 binding proteins. The yeast reporter strain (Y190) contained the *lacZ* reporter gene with the Gal1 binding site as the upstream activating sequence (UAS_G) as indicated. Expression of the Gal4 protein results in recognition of the UAS_G by its DNA binding domain (DBD) and activation of the *lacZ* reporter gene by its acidic activation domain (AD) (top left). Expression of the Gal4DBD alone results in binding of the DBD to the UAS_G but no reporter activation. Expression of the AD alone does not activate *lacZ* as it is not recruited to the *lacZ* gene. Ku80 fragments were expressed as Gal4DBD fusions in combination with clones from a human Jurkat T-cell cDNA library (represented by X) fused to the Gal4AD. Recruitment of the Gal4DBDKu80 construct to the reporter gene is through the interaction of the Gal4DBD with its recognition sequence as shown to the right. An interaction between Ku and library clones result in recruitment of the Gal4AD upstream of the minimal promoter and transcriptional induction of the *lacZ* reporter gene (right).

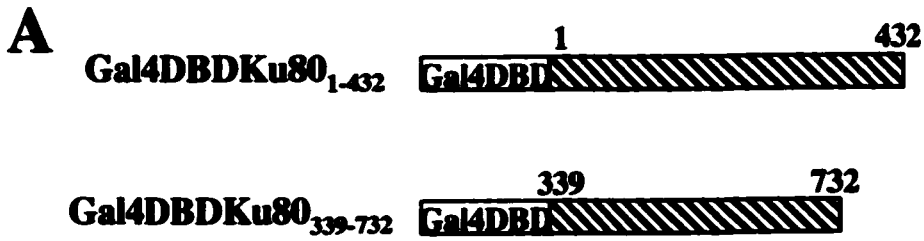

Gal4
ON
UASG
LacZ
DBD
OFF
UASG
AD
OFF
UASG
ON
UASG

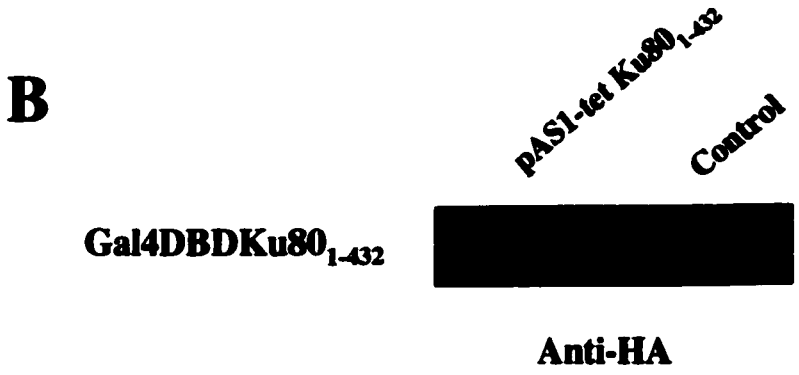
In performing the yeast-two hybrid screening with a plasmid containing smaller portions of Ku80, I did not observe plasmid recombination.

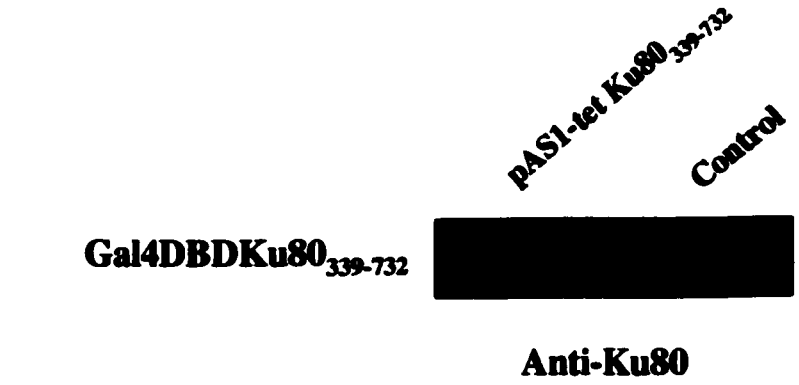
The library was independently screened with two overlapping Gal4 DNA binding domain-Ku80 fragments comprising amino acids 1-432 and 339-732 of the N and C-terminus of Ku80, respectively. Shown in Figure 34A is a schematic illustration of the Ku80 constructs. Figure 34B shows Western blots demonstrating expression of both Gal4DBDKu80 proteins in the yeast strain (Y190). The Y190 yeast strain used for the two-hybrid screening has the *lacZ* and *HIS3* reporters. Both reporter genes contain the Gal1 upstream activating sequence. Expression of the Gal4DBDKu80$_{1-432}$ or Gal4DBDKu80$_{339-732}$ constructs in this strain will result in binding of the Ku80 fragments to the Gal1 UAS mediated by the Gal4DBD.

For library screening a Gal4DBDKu80 construct is co-expressed with library clones that are fused to the Gal4 activation domain. An interaction of the Gal4DBDKu80 construct with a library protein will result in the library protein being recruited to both the *lacZ* and *HIS3* reporter genes and subsequent activation of the reporter genes by the Gal4 activation domain. Stimulation of *lacZ* transcription was determined by the β-galactosidase filter assay. *HIS3* activation would be detected by enhanced growth of the yeast colony in the absence of histidine. However, since the *HIS3* reporter in this particular yeast strain exhibited spurious basal activity the yeast two-hybrid screening was performed in the presence of the His3 protein competitive inhibitor 3-amino-1,2,4-triazole (3-AT) that significantly retards the growth of the yeast in the absence of *HIS3* reporter activation. Therefore, yeast with a Ku80 interacting clone would result

Figure 34. Schematic illustration and expression of the Gal4DBDKu80 constructs used to screen the Jurkat T-cell cDNA library for putative Ku80 binding proteins using the yeast two-hybrid system. (A) Schematic illustration of the overlapping N and C-terminal fragments of the Gal4DBDKu80 constructs representing amino acids 1-432 and 339-732 of Ku80, respectively. (B) Western blots showing expression of the Gal4DBDKu80 proteins in yeast. The Y190 strain was transformed to express either the N or C-terminal Gal4DBDKu80 construct or transformed with empty parent plasmids (control) as shown at the top. Yeast colonies were grown for 5 days at 30 °C on appropriate solid synthetic dropout medium. A yeast colony was harvested and grown in 5 ml of liquid culture at 30 °C to mid-log phase. Yeast were washed and then heated to 95 °C for 5 min in the presence of 2XSDS sample buffer to lyse the cells. The extracts were centrifuged for 5 min at RT and the supernatants were run on a 10 % SDS-PAGE gel. The proteins were transferred to membrane and the Gal4DBDKu80 constructs were detected using a HA-tag antibody (top) or a Ku80-specific antibody (111) (bottom).


A
1
432
Gal4DBDKu80$_{1-432}$
Gal4DBD
339
732
Gal4DBDKu80$_{339-732}$
Gal4DBD


B
pAS1-tet Ku80$_{1-432}$
Control
Gal4DBDKu80$_{1-432}$
Anti-HA


pAS1-tet Ku80$_{339-732}$
Control
Gal4DBDKu80$_{339-732}$
Anti-Ku80

in a large colony (*HIS3* activation) that turns blue in a β-galactosidase filter assay (*lacZ* activation).

I screened approximately 240,000 and 300,000 yeast colonies with Gal4DBDKu80$_{1-432}$ and Gal4DBDKu80$_{339-732}$, respectively. Although addition of 3-AT reduced the number of yeast colonies observed following transformation with Ku80 and library clones, it did not completely eliminate colony formation despite absence of *HIS3* reporter activation. However, the number of colonies observed in the presence of 3-AT was determined to be approximately 4.5 times less than in its absence. Since a positive interaction would result in a detectable, large yeast colony I actually screened an estimated 1, 080,000 and 1,350,000 library clones with Gal4DBDKu80$_{1-432}$ and Gal4DBDKu80$_{339-732}$, respectively. Given that the library contained an estimated 1 million independent clones, a large proportion of these clones were tested for an interaction with Ku80. Unfortunately, I found no library clones that interacted with either Gal4DBDKu80$_{1-432}$ or Gal4DBDKu80$_{339-732}$. A complete summary of the yeast two-hybrid results is presented in Table 2.

Given that Ku has been implicated in numerous cellular processes it was expected that several Ku80 interacting proteins would be identified using the yeast two-hybrid system. The failure to detect any interacting clones was surprising. However, most of the proteins that have been reported to interact with Ku to date, interact with Ku through the Ku70 subunit. Since the large majority of Ku binding proteins interact with Ku70, it may reflect the greater accessibility of Ku70 to bind proteins in the context of the heterodimer. Consistent with this is the recent crystal structure of Ku that shows that the

Table 2. Summary of the yeast-two hybrid screening of the Jurkat T-cell cDNA library with Ku80 fragments. The Y190 yeast strain was used to screen over an estimated 1 million clones from a Jurkat T-cell cDNA library for putative Ku80 binding proteins with both Gal4DBDKu80$_{1-432}$ and Gal4DBDKu80$_{339-732}$ constructs. Overlapping N and C-terminal Ku80 fragments fused to the Gal4 DNA binding domain representing amino acids 1-432 and 339-732, respectively, were use to independently screen the library. Shown are the results obtained from the screening using the Gal1-*GAL1-lacZ* reporter gene. The 3-amino-1,2,4-triazole (3-AT) factor is also indicated. No Ku80 interacting clones were identified.

Table 2

Gal4DBDKu80 Construct	Number of Yeast Colonies Screened	3-AT Factor	Number of Library Clones Screened	Positive Clones (First Screen)	Negatives Clones	False Positives Clones	Positives Clones
$Ku80_{1-432}$	240,000	4.5	1,080,000	27	9	18	0
$Ku80_{339-732}$	300,000	4.5	1,350,000	43	11	32	0

C-terminal arm of Ku70 extends out from the ring structure of the Ku heterodimer. The greater accessibility of this region may have evolved for interaction with other proteins. Our lab has shown that this region of Ku70 mediates the interaction of Ku with several homeodomain proteins.

Surprisingly at the time, I also failed to obtain Ku70 as a Ku80 interacting clone in the yeast two-hybrid screening. The most likely explanation for this result is that Ku70 expressed with a Gal4 activation domain is able to activate reporter genes on its own, as I have shown in the first part of my thesis. I determined that ADKu70 activates the *lacZ* reporter of the Y190 strain used in the two-hybrid screening. Therefore, Gal4ADKu70 library clones would have been categorized as false positives in the screening.

APPENDIX D

Base composition of the promoters activated by Ku Antigen

CYC1

aagcttgaat tcgagctcgg tacccgggga tctgtcgacc tcgaggcatg tgctctgtat
gtatataaaa ctcttgtttt cttcttttct ctaaatattc tttccttata cattaggtcc
tttgtagcat aaattactat acttctatag acacgcaaac acaaatacac acactaaatt
aata

HIS3

gaattcccgg ggagctcacg cgttcgcgaa tcgatccgcg gtctagaaat tcctggcatt
atcacataat gaattataca ttatataaag taatgtgatt tcttcgaaga atatactaaa
aaatgagcag gcaagataaa cgaaggcaaa g

GAL1

ggaaacagct atgaccatga ttacgccaag cgcgcaatta accctcacta aagggaacaa
aagctgggta ccgggccccc cctcgagcgg aattcttact tcttattcct ctaccggatc
ccgctcgagg tcgacccacg cgtccgaaaa aaaagggcgg ccgccaccgc ggtggagctc
caattcgccc tatagtgagt cgtattacgc gcgctcactg gccgtcgttt tacaa

Curriculum Vitae

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AWARDS and SCHOLARSHIPS:

Ontario Graduate Scholarship – Sept. 2001-Dec-2001
University of Ottawa Award of Excellence – Sept. 2001-Dec. 2001
Natural Science and Engineering Research Council Scholarship (PGS-B) – Sept. 1999-01
University of Ottawa Award of Excellence – Sept. 1999
University of Ottawa Admission Scholarship – Sept. 1998-99
Ontario Gradate Student Science and Technology Scholarship – Sept. 1998-99

PUBLICATIONS:

Nuclear localization of Ku antigen is promoted independently by basic motifs in the Ku70 and Ku80 subunits. **Jesse Bertinato,** Caroline Schild-Poulter, Robert J.G. Haché. (*Journal of Cell* Science 2001; 114, 89-99.)

Well-Defined Regions of Apolipoprotein B-100 Undergo Conformational Change During Its Intravascular Metabolism. Xingyu Wang, Richard Pease, **Jesse Bertinato**, Ross W. Milne. (*Arterioscler. Thromb. Vasc. Biol.* 2000; 20:1301-1308.)

Plasma Lipoproteins And Their Receptors. Robert Raffai, Xingyu Wang, Vinita Chauhan, Tahereh Ejtehadian, **Jesse Bertinato** and Ross Milne. (*Interscientia* 1996.)

Evidence Implicating Ku Antigen as a Structural Factor in RNA Polymerase II-Mediated Transcritpion. **Jesse Bertinato,** Julianna Tomlinson, Caroline Schild-Poulter, Robert J.G. Haché. (In preparation)

Interaction of Ku Antigen with TATA Box Binding Protein is Stabilized by DNA. **Jesse Bertinato,** Caroline Schild-Poulter, Robert J.G. Haché. (In preparation)

Distinct Pathways Regulate the Growth Rate and the Temperature Sensitivity of Ku-Deficient *Saccharomyces cerevisiae*. **Jesse Bertinato,** Caroline Schild-Poulter, Robert J.G. Haché. (In preparation)

ABSTRACTS PRESENTED:

AACR Annual meeting March 28-April 1, 1998 New Orleans, LA-Discrimination of the Sequence-Specific and DNA-End Binding Activities of Ku Antigen. **Bertinato, J.P.,** Schild-Poulter, C., and Haché, R.J.G.

CSHL meeting in June 1998-Sequence-Specific DNA-dependent Protein Kinase activity and the repression of Mouse Mammary Tumour Virus Transcription requires an extended Ku Antigen DNA recognition sequence. Ward Griffin, **Jesse Bertinato**, and Robert Haché.

World Congress of Cellular and Molecular Biology-Jena, Germany Oct. 8-13, 2000-Nucleo-cytoplasmic Trafficking of the Ku Autoantigen. **Jesse Bertinato**, Rhian Walther, Caroline Schild-Poulter and Robert J.G. Haché.

SPECIALIZED SKILLS:

Molecular Biology: cloning, site-directed mutagenesis, PCR, southern, Northern and Western blotting, generation and expression of recombinant proteins in bacteria, yeast, and mammalian cells, EMSA, ELISA, cell culture, immunoprecipitation of proteins, analysis of DNA fragmentation during apoptosis, FACs analysis, *in vivo* cell labeling, protein phosphorylation, generation of polyclonal and monoclonal antibodies, measuring antibody affinities

Fluorescence Microscopy: confocal microscopy, direct fluorescence on live cells, indirect immunofluorescence, fluorescence recovery after photobleaching (FRAP)

Yeast Genetics: generating yeast knockout strains, transforming yeast, generating yeast reporter strains, analyzing temperature sensitive and growth phenotypes, analyzing transcription of yeast genes, yeast one-hybrid and two-hybrid analysis

Contribution of Collaborators

Julianna Tomlinson constructed the pET-30a(+)Ku70 plasmid and played a large role in generating the pBJ5Ku70$_{58-609}$ plasmid and performing the biochemical fractionation experiments shown in Figure 11.

Rhian Walther performed the photobleaching experiments presented in Figure 31.

Caroline Schild-Poulter generated the pACTIIKu70$_{1-609}$, pACTIIKu70$_{1-99}$ and pActIIKu80 plasmids.

Anna Su produced and purified the 6xHisKu70 protein from bacteria used in Figure 26B.