

# Ku86 Variant Expression and Function in Multiple Myeloma Cells Is Associated with Increased Sensitivity to DNA Damage<sup>1</sup>

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Ku is a heterodimer of Ku70 and Ku86 that binds to double-stranded DNA breaks (DSBs), activates the catalytic subunit (DNA-PKcs) when DNA is bound, and is essential in DSB repair and V(D)J recombination. Given that abnormalities in Ig gene rearrangement and DNA damage repair are hallmarks of multiple myeloma (MM) cells, we have characterized Ku expression and function in human MM cells. Tumor cells (CD38<sup>+</sup>CD45RA<sup>-</sup>) from 12 of 14 (86%) patients preferentially express a 69-kDa variant of Ku86 (Ku86v). Immunoblotting of whole cell extracts (WCE) from MM patients shows reactivity with Abs targeting Ku86 N terminus (S10B1) but no reactivity with Abs targeting Ku86 C terminus (111), suggesting that Ku86v has a truncated C terminus. EMSA confirmed a truncated C terminus in Ku86v and further demonstrated that Ku86v in MM cells had decreased Ku-DNA end binding activity. Ku86 forms complexes with DNA-PKcs and activates kinase activity, but Ku86v neither binds DNA-PKcs nor activates kinase activity. Furthermore, MM cells with Ku86v have increased sensitivity to irradiation, mitomycin C, and bleomycin compared with patient MM cells or normal bone marrow donor cells with Ku86. Therefore, this study suggests that Ku86v in MM cells may account for decreased DNA repair and increased sensitivity to radiation and chemotherapeutic agents, whereas Ku86 in MM cells confers resistance to DNA damaging agents. Coupled with a recent report that Ku86 activity correlates with resistance to radiation and chemotherapy, these results have implications for the potential role of Ku86 as a novel therapeutic target. *The Journal of Immunology*, 2000, 165: 6347–6355.

**H**uman multiple myeloma (MM)<sup>3</sup> is characterized by the monoclonal expansion of plasma cells in the bone marrow (BM). Although MM is initially responsive to therapy, drug resistance evolves in most patients, and this disease remains incurable. Novel biologically based therapies are urgently needed. At present, the development of MM is believed to proceed through a multiple-step transformation (1). Recent elegant studies of myeloma cell lines and freshly isolated tumor cells have shown chromosomal translocations involving the switch regions of the Ig heavy-chain (IgH) locus and multiple partner chromosomes (2). However, an underlying central molecular abnormality to account for chromosomal instability in MM has not to date been identified.

Double-stranded DNA breaks (DSBs) are generated during initiation of meiotic recombination or V(D)J recombination of Ig and TCR genes. Ionizing radiation (IR) and certain chemotherapeutic drugs also induce DSBs. Due to the instability of DSBs and disruption of the genome, highly efficient mechanisms have evolved for both recognition and repair of DSBs. Specifically, DSBs are repaired in mammalian cells primarily via nonhomologous or il-

legitimate recombination (3, 4), which is dependent upon Ku/DNA-dependent protein kinase (DNA-PK) (5, 6). Ku, originally identified as a target Ag in a patient with autoimmune disease (7), is an abundant nuclear protein that binds selectively to free double-stranded DNA (8). It consists of Ku70 and Ku86 subunits that bind with high affinity to altered DNA, including DSBs (9, 10). Ku is the regulatory subunit of DNA-PK and is required for DNA-PK activity (11): Ku binds to DSBs, recruits the catalytic subunit of the 460-kDa protein DNA-PKcs, and activates its kinase function (12) to phosphorylate DNA-bound proteins (13). Cells deficient in Ku86 are hypersensitive to IR in vitro, due to defects in DSB rejoining (14); conversely, transfection of human or hamster Ku86 gene into Ku86 mutant cells can restore DSB repair (15–17). Ku-deficient cells are also deficient in DNA-PK activity, further suggesting that Ku DNA-binding is required for DNA-PK activation in vivo (18). The observation that Ku86 (19, 20) or Ku70 (21) knockout mice demonstrate hypersensitivity to IR further confirms the role of Ku in DSB repair in vivo. Ku86 has also been shown to be required for normal Ig rearrangement and IgH class switch recombination (22, 23). This may be relevant in MM because chromosome translocations involving the IgH switch regions are common. In addition, Ku80 has been implicated in maintaining the integrity of the genome via suppression of chromosomal rearrangements (24).

Despite many studies with established cell lines, little is known about Ku activity and its regulation in fresh human cells and tissues. Studies in resting murine B cells showed low levels of Ku, and that Ku expression could be up-regulated by culturing the cells with CD40L and IL-4 (25). In addition, Ku was found in the cytoplasm without any identified biological function (25, 26). Altered forms of Ku86 with lesser m.w. have also been described (27–29). We have previously demonstrated ectopic localization of Ku86 in the cell membrane of human MM cells after CD40L activation (30). The Ku86 on the membrane of activated MM cells

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<sup>3</sup> Abbreviations used in this paper: MM, multiple myeloma; DSBs, double-stranded DNA breaks; WCE, whole cell extracts; DEB, DNA end binding; CM, complete medium; BM, bone marrow; IR, ionizing radiation; NBM, normal BM; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit.

mediates both homotypic tumor cell adhesion and heterotypic adhesion of tumor cells to fibronectin and BM stromal cells. These results, coupled with the abnormalities in DNA repair and Ig gene rearrangement characteristic of human MMs, suggest that abnormalities in the Ku86 may play a role in the biology of MM.

In this study, we characterized the expression and function of Ku86 in human MM. We demonstrate that a variant of Ku86 (Ku86v) is expressed in freshly prepared tumor cell lysates from a majority of MM patient BM aspirates. Immunoblotting studies show reactivity with Abs for Ku86 N terminus, but not with Abs directed against Ku86 C terminus, suggesting C terminus truncation. EMSA with these Abs confirmed truncation of C terminus in Ku86v and further demonstrated decreased DNA end binding (DEB) activity and DNA-PKcs binding, resulting in a lack of detectable DNA-PKcs complexes and kinase activity. 5' end Ku86 transcripts are detectable in all samples, whereas 3' end Ku86 transcripts are somehow altered or reduced in patients expressing only Ku86v protein, suggesting posttranscriptional modification. Finally, patient MM cells expressing Ku86v exhibit hypersensitivity to DNA-damaging agents, including mitomycin C, bleomycin, and  $\gamma$  irradiation compared with patient MM cells or normal BM (NBM) donors with normal Ku86. Therefore, these studies provide mechanisms for both decreased DSB repair and sensitivity to DNA damaging agents in those MM cells expressing Ku86v, and further suggest that Ku86 in MM cells may confer relative resistance to treatment and, therefore, represent a novel therapeutic target.

## Materials and Methods

### BM cell preparation

BM aspirates from patients with MM ( $>70\%$  CD38<sup>+</sup>CD45RA<sup>+</sup> cells) were collected in heparinized tubes following Institutional guidelines with informed consent. Suspensions enriched for tumor cells were prepared by density gradient centrifugation (Ficoll-Hypaque; Pharmacia, Uppsala, Sweden) followed by depletion of erythrocytes and non-MM cells using immunomagnetic bead separation (31). Briefly, cell suspensions were incubated with a cocktail of mouse mAbs directed against CD3 (T cells); CD11b and CD14 (monocytes); CD33 (myeloid cells); CD45 and CD45RA (leukocyte common Ag); CD32 (Fc $\gamma$ RIIA); as well as glycoporphin A (erythrocytes) for 30 min in the cold room, with constantly gentle agitation. Then, non-MM cells were removed by incubation with goat anti-mouse IgG (Fc) Ab coupled to immunomagnetic beads (Dynabeads M450; Dynal, Oslo, Norway). Resultant populations were  $>95\%$  MM cells (CD38<sup>+</sup>CD45RA<sup>+</sup>) and  $\geq 98\%$  viable, assessed by trypan blue exclusion. MM cells were also  $>95\%$  positive for CD138 or syndecan-1 (32, 33). Cells were resuspended in complete medium (CM: RPMI 1640 media containing 10% heat-inactivated FBS, 25 IU/ml penicillin, 25  $\mu$ g/ml streptomycin, and 2 mM L-glutamine) for further analysis.

After informed consent, NBM was obtained from healthy donors at the time of allogeneic BM harvest. Mononuclear cell suspensions separated by Ficoll-Hypaque gradient centrifugation were resuspended in CM ( $1 \times 10^6$  cells/ml) and incubated in tissue culture flasks for 1 h at 37°C. Monocytes were removed by adherence, and the resultant lymphocyte cell suspensions contained  $\leq 0.5\%$  CD14<sup>+</sup> cells with  $>95\%$  viability. They were immediately used as controls for further analysis, as described below.

### Cell culture

Cell lines (CESS, Jurkat, 293) (American Type Culture Collection, Manassas, VA) were grown at 37°C in CM. IR was performed at room temperature using a Gammacell-1000 (Atomic Energy of Canada, Ottawa, ON, Canada) under aerobic conditions with <sup>137</sup>Cs source emitting at a fixed dose rate of 300 rad/min.

### Cell extract preparation

To prepare whole cell extracts (WCE), cells were washed in PBS, pelleted, and resuspended in extraction buffer: 0.5% Nonidet P-40; 20 mM HEPES pH 8.0; 20% glycerol (v/v); 400 mM NaCl containing 0.5 mM DTT; 0.2 mM EDTA; 1  $\mu$ g/ml aprotinin; 10  $\mu$ g/ml leupeptin; 0.5 mM PMSF; 1.5  $\mu$ g/ml pepstatin with 0.5  $\mu$ g/ml proteinase inhibitors (proteinase inhibitor cocktail tablets; Boehringer Mannheim, Indianapolis, IN, and Calbiochem, La Jolla, CA); as well as phosphatase inhibitors, 50 mM NaF and 1 mM

Na<sub>3</sub>VO<sub>4</sub>. pH was carefully maintained to assure inhibitor activity. We also performed experiments to lyse MM cells directly in SDS lysing buffer (62.5 mM Tris-Cl, pH 7.0, 2% SDS, 720 mM 2-ME, 5 mg bromophenol blue) and boiled samples for 5 min, a process that inactivates proteases. After incubation on ice for 30 min and microcentrifugation at 4°C, supernatants were transferred to new microfuge tubes. Protein concentrations were determined using Bradford assay (Bio-Rad, Hercules, CA) and samples aliquoted and stored at  $-80^\circ\text{C}$ .

### Western blot analysis

Protein lysates were subjected to electrophoresis on a 6 or 8% SDS-PAGE. Routinely, 15  $\mu$ g of WCE from each sample was then transferred into polyvinylidene difluoride membrane (Millipore, Freehold, NJ) and the membrane blocked with TBST/5% milk. The membrane was next hybridized with Ab overnight in the cold room, washed, and then incubated with anti-mouse IgG-HRP Ab (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The reaction was visualized using the ECL system (DuPont-NEN, Boston, MA). Abs used in Western blotting experiments were as follows: murine anti-Ku mAbs 111 (antiKu86 aa 610–705), S10B1 (antiKu86 aa 8–221), NC91 (antiKu86 aa 1–374), N3H10 (antiKu70 aa 506–541), anti-DNA-PKcs mAb 18–2 (aa 1–2713), anti-DNA-PKcs (aa 42–27) (Labvision, Fremont, CA), 5E2 (antiKu86 aa 535–543) (30), anti- $\alpha$ -tubulin DM1A mAb (Sigma, St. Louis, MO), and B-B4 mAb (syndecan-1; Serotec, Raleigh, NC).

### DEB assay

EMSA was performed as described previously (27, 34) to determine Ku-DEB activity and complex formation, specifically to define whether the Ku86v-DNA protein complexes demonstrate distinct electrophoretic mobility. To obtain the radiolabeled DNA probe, two 25 mer-oligonucleotides (5'-ACTTGATTAGTTACGTAACGTTATG-3' and 5'-CATAACGTTACGTAACCTAATCAAGT-3') were annealed and end-labeled with T4 polynucleotide kinase in the presence of [ $\gamma$ -<sup>32</sup>P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq; NEN, Cambridge, MA). The probes were purified by chromatography through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ).

The radiolabeled 25-mer DNA probe (4 ng; 100,000 cpm) was incubated with WCE (1  $\mu$ g) in 15  $\mu$ l of binding buffer (10 mM Tris/pH 8.0, 1 mM EDTA, 10% glycerol, 50 mM KCl, 0.5 mM PMSF) for 20 min on ice, in the presence of 1  $\mu$ g of unlabeled supercoiled plasmid DNA to compete for nonspecific DNA-binding proteins. The protein-bound and free oligonucleotides were electrophoretically separated on 5% native polyacrylamide gels at room temperature for 3 h at 130 V in 0.5 $\times$  TBE (90 mM Tris-borate, 2 mM EDTA) running buffer. Gels were dried and exposed to X-OMAT films (Eastman Kodak, Rochester, NY) at  $-80^\circ\text{C}$ . For gel mobility supershift experiments, mAbs against Ku70/Ku86 heterodimer (162), Ku86 (111, S10B1), or BAX (rabbit polyclonal Ab as a control Ab unreactive with Ku86; PharMingen, San Diego, CA) were preincubated with WCE and added to binding mixtures. To control for DNA binding activities in the same cell extracts, EMSA was also conducted in the absence of nonspecific competitor DNA (either plasmids or poly(dI:dC)).

### DNA-PK activity assay

The DNA-PK "pull down" kinase assay was performed as previously described (18). WCE prepared as described above were tested for DNA-PK activity by first absorbing protein onto double-stranded DNA cellulose beads, which was then assayed for ability to phosphorylate a p53 peptide substrate. Each sample was assayed in the presence of either wild-type peptide or mutated peptide, as well as in the absence of peptide as negative controls. In brief, 150  $\mu$ g of WCE was incubated with 50  $\mu$ l of dsDNA cellulose (30 mg/ml) in 1 ml K buffer (25 mM Tris-HCl, pH 7.9, 10 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 2.5% glycerol) containing 60 mM NaCl for 30 min at 4°C. The DNA cellulose was then washed by centrifugation three times in 1 ml of Z'0.05 buffer (25 mM HEPES pH 7.9, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 20% glycerol, 0.1% Nonidet P-40, 1 mM DTT) and resuspended in 50  $\mu$ l of Z'0.05 buffer. An aliquot (15  $\mu$ l) of the DNA cellulose was assayed for DNA-PK activity by adding 0.5  $\mu$ l of [ $\gamma$ -<sup>32</sup>P]ATP (300  $\mu$ Ci/mmol; 1 Ci = 37 GBq) in the presence of 4 nmol (0.2 mM) of synthetic DNA-PK peptide substrates derived from the N-terminal transcriptional activation domain of murine p53 (wild-type peptide, EPPLSQEAFADLLKK; mutated peptide, EPPLSEQAFADLLKK). Reactions were stopped and analyzed by spotting onto phosphocellulose paper, washing, and liquid scintillation counting. DNA-PK activity for a given WCE was expressed in cpm incorporated in the wild-type or mutated peptide minus the background signal in the absence of peptides. To determine the status of Ku86, Ku70, and DNA-PKcs in the kinase reaction, an aliquot of beads was washed with Z'0.05 buffer and incubated for 1 h in 50 mM

Tris-HCl (pH 9.0)/Triton 1%, with constant agitation to elute proteins bound to the beads. The eluted proteins were electrophoresed in 7% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane, which was subjected to Western blot analysis for the presence of DNA-PKcs, Ku86, and Ku70 proteins.

#### Radiation/drug sensitivity assay

Highly viable (98%, assessed by trypan blue exclusion) MM (CD38<sup>+</sup>CD45RA<sup>-</sup>) cells that either express both wild-type Ku86 and Ku86v or Ku86v alone were isolated and suspended in CM. Cells with only full-length Ku86 (as NBM) were used as positive control. Cells were treated with  $\gamma$  irradiation (0–10 Gy), mitomycin C (0–5  $\mu$ g/ml) (Ben Venue Laboratories, Bedford, OH), or bleomycin (0–5  $\mu$ g/ml) (Blenoxane; Bristol-Myers Squibb, Princeton, NJ) on day 0. Both control (untreated) and treated MM cells (1–2  $\times 10^6$  cells/ml) were seeded into 96-well microculture plates (200  $\mu$ l/well) and incubated at 37°C in a 5% CO<sub>2</sub> incubator. Quadruplicate experiments were performed at each treatment dose. The sensitivity/cytotoxicity assays were used to define viable tumor cells remaining after treatment. Specifically, viable cells were enumerated in control vs treated cultures using trypan blue exclusion 2 days after treatment. In contrast, apoptosis in MM cells was assessed using annexin V-FITC (PharMingen, San Diego, CA) staining, and FACS analysis. When it is possible, MM cells were cultured for 24 h after treatment, incubated with 1  $\mu$ Ci of tritiated thymidine ([<sup>3</sup>H]TdR; DuPont-NEN) for 18 h, harvested onto glass filters using the Harvester 96 MACH II harvester (Tomtec, Orange, CT), and analyzed on the 1205 Betaplate  $\beta$ -counter (Wallac, Gaithersburg, MD).

#### Northern blot analysis

Total cellular RNA from patient MM cells (CD38<sup>+</sup>CD45RA<sup>-</sup>) was extracted by RNeasy kit (Qiagen, Valencia, CA). Five micrograms of total cellular RNA from each sample were separated on a 1.2% agarose-formaldehyde gel, transferred to Nitropure membrane (Micron Separation, Westboro, MA) in 10 $\times$  SSC, and hybridized with <sup>32</sup>P-labeled probes. Two cDNA probes were used that were specific for 5' and 3' Ku86 RNA, respectively. They were obtained through RT-PCR using primer pairs (5'-1/5'-2; 3'-1/3'-2) as described below. The resultant 5' probe (1097 bp) and 3' probe (1082 bp) were purified and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP to ~10<sup>9</sup> cpm/ $\mu$ g of specific activity using the random primer method. An 18S rRNA oligonucleotide, 5' ACGGTATCTGATCGTCTTCGAACC 3' with <sup>32</sup>P end labeling was used as the loading control. Hybridization was conducted at 68°C in QuickHyb (Stratagene, La Jolla, CA) in the presence of 100  $\mu$ g/ml of salmon sperm DNA. After washing, the blots were dried and analyzed by autoradiography.

#### Sequencing for Ku86 cDNA

Total cellular RNA (2.5  $\mu$ g) was used for 20- $\mu$ l reverse transcription reaction with Random Primer and Superscript II RNaseH<sup>-</sup> reverse transcriptase (Life Technologies, Gaithersburg, MD). RT (2  $\mu$ l) was used for RT-PCR on the TC-480 thermocycler (Perkin-Elmer, Norwalk, CT) using this

program: 94°C for 3 min followed by 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min and an extension at 72°C for 7 min. Each RT-PCR product was directly sequenced using Dye Terminator Cycle Sequencing Kits and an ABI Prism 377 DNA Sequencer (Perkin-Elmer). Both sense and antisense primers were used to confirm the fidelity of the sequence. Primers used in this study were as follows (schematic diagram shown in Fig. 8A): 5'-1: 5'-TTGTACAGCGACAGGTGT-3'/5'-2: 5'-GCCGACTTGAGGATTAGC-3'; 3'-1: 5'-CTCCCTGATTCATGCTTT-3'/3'-2: 5'-CCCATACATCCACGACCT 3'; F2: 5'-ATGTGCAGCTGCCTTTCATGGAAG-3'/B2: 5'-TTTGTCTTTGGGGGCCAGAACTT-3'; F3: 5'-GCTGTTGATGCTTTGATTGACTCC-3'/B3: 5'-TGCTGTGTCTCCA CTGGTTTG-3'. As a control for RT-PCR, a primer pair to amplify a 630-bp fragment of  $\beta$ -actin was used: upper primer: 5'-TCACCCACACTGTGC CAT-3' and lower primer: 5'-GCATTTCGCGTGGACGATG-3'.

These primers allowed the amplification of sequences from coding regions of Ku86 cDNA.

#### Protein microsequencing and mass spectrometry

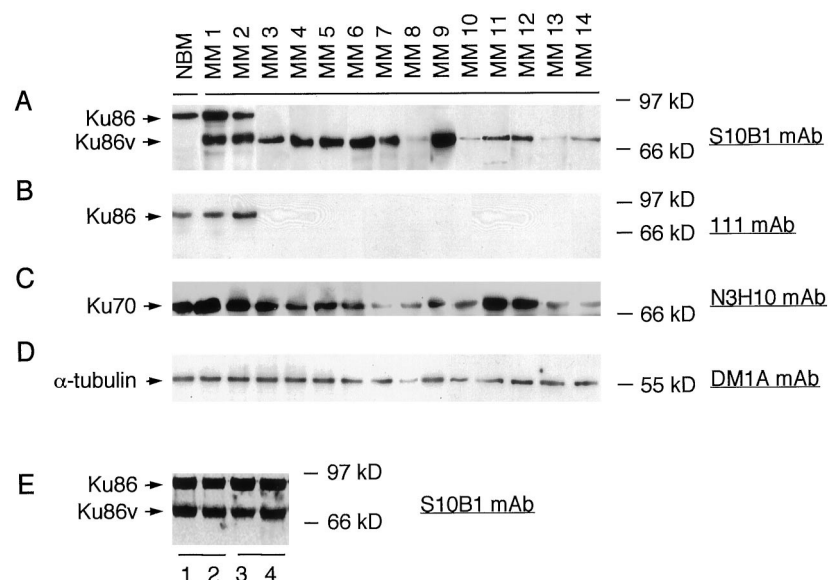
Protein microsequencing and mass spectrometry were performed by Harvard Microchemistry Facility at Harvard University (Cambridge, MA). In brief, WCE from MM with only Ku86v expression were prepared as described before, incubated with N terminus Ku86 Ab (S10B1 mAb or 5E2 mAb), and then overnight with protein A-Sepharose (Sepharose CL-4B; Amersham Pharmacia Biotech, Piscataway, NJ). The immunopurified protein was washed and then resolved on 8% SDS-PAGE, followed by staining with Coomassie Brilliant Blue R-250 (CBB) (Bio-Rad). Protein species at the expected m.w. were dissected out from the gel for further proteolytic digestion. Peptide sequence analysis was then performed by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry ( $\mu$ LC/MS/MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer.

## Results

#### Ku86v is expressed in patient MM cells

To characterize Ku86 expression in MM patients, WCE from patient MM (>97% CD38<sup>+</sup>CD45RA<sup>-</sup>CD138<sup>+</sup>) cells were immunoblotted with Ku86-specific Abs. NBM served as a control for the expression of Ku86. As shown in Fig. 1A, immunoblotting of MM patient lysates with Ab directed at Ku86 N terminus (S10B1: aa 8–221) demonstrated a 69-kDa protein that was not detected in NBM. Twelve of fourteen (86%) MM patient samples expressed only this 69-kDa protein and lacked Ku86 (Fig. 1A, MM 3–14), whereas two patients expressed both the 69-kDa protein and Ku86 (MM 1–2). Immunoblotting of six additional NBM samples from healthy donors detected only normal Ku86 (data not shown). Immunoblotting with anti-Ku70 Ab detected Ku70 in all samples, including those patient MM cells lacking normal Ku86 (Fig. 1C).

**FIGURE 1.** Ku86v is expressed in patient MM cells and has truncated C terminus. A, WCE (15  $\mu$ g/lane) of either NBM (lane 1) or patient MM (CD38<sup>+</sup>CD45RA<sup>-</sup>) cells (lanes 2–15) were immunoblotted with the following mAbs: S10B1 (Ku86, aa 8–221) (A); 111 (Ku86, aa 610–705) (B); N3H10 (Ku70, aa 506–541) (C); and DM1A ( $\alpha$ -tubulin) (D). E, Immunoblotting using S10B1 mAb of cell lysate preparations was performed, in the presence of protease inhibitors (lanes 1 and 2) and in SDS-PAGE sample buffer without protease inhibitors (lanes 3 and 4). Lanes 1 and 3 are MM cells of an individual patient, whereas lanes 2 and 4 are MM cells from another patient. Protein markers are at 66 and 97 kDa.

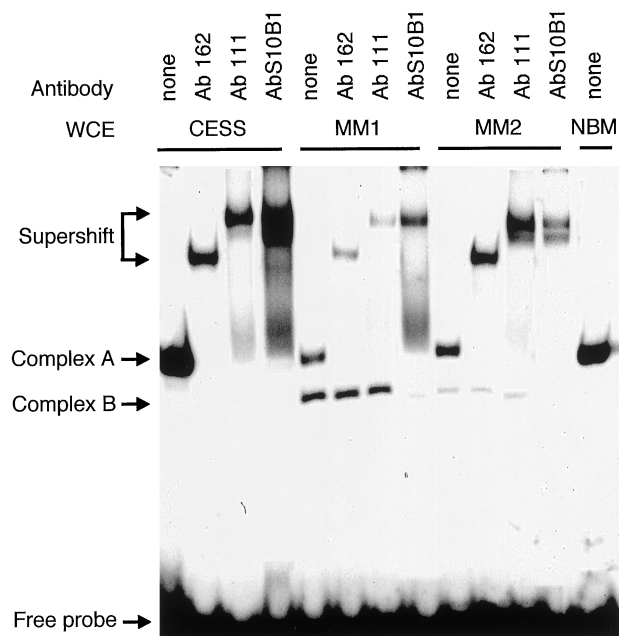


To further characterize this 69-kDa protein, immunoblotting of WCE of patient MM cells and NBM was performed using mAbs targeting distinct Ku86 epitopes. Ab directed to Ku86 N terminus (S10B1) was immunoreactive with Ku86 in the NBM control, both Ku86 and Ku86v in two MM patient samples (MM 1 and 2), and Ku86v only in the remainder of patient MM cells (Fig. 1A, MM 3–14). Immunoblotting with other Abs targeting Ku86 N terminus (NC91: aa 1–374 and 5E2: aa 535–543) demonstrated a similar pattern of reactivity (data not shown). In contrast, immunoblotting with mAb directed at Ku86 C terminus (111) precipitated Ku86 in NBM control and in two MM patient samples (MM 1 and 2), but no reactivity was noted in those patient MM cells expressing only Ku86v (MM 3–14) (Fig. 1B). Moreover, increased loading of WCE (75–100  $\mu$ g/lane) before immunoblotting with mAb 111 did not alter results. Because Ku86 associates with Ku70, we also examined the expression of Ku70 in these WCE. Immunoblotting with mAb N3H10, which targets aa 506–541 of Ku70, demonstrated Ku70 in NBM and all patient MM cells (Fig. 1C). Immunoblotting with  $\alpha$ -tubulin Ab further confirmed integrity of protein lysates (Fig. 1D). Finally, to ensure that the Ku86v is expressed in vivo and not the product of in vitro proteolysis during lysate preparation, MM cells (CD38<sup>+</sup>CD45RA<sup>−</sup>) from two patients were divided into two aliquots. Cell lysates from the first aliquot in each case were prepared in the presence of protease inhibitors, as described in *Materials and Methods*. Cell lysates were prepared from the second aliquot of MM cells from each patient directly in SDS-PAGE sample buffers. Immunoblotting was performed using mAb against Ku86 N terminus (S10B1). As shown in Fig. 1E, the expression of Ku86v was similarly detected in the presence or absence of protease inhibitors. In addition, the ratio of Ku86 to Ku86v was not changed either in the presence or absence of protease inhibitors. Similar results were obtained using another Ku86 N terminus mAb 5E2 (data not shown).

#### *Ku86v in patient MM cells exhibits differential electrophoretic mobility*

Ku binds to double-stranded (ds) DNA ends, which is required for formation of Ku-DNA-PK complex formation and kinase activity. Therefore, we first tested the binding activity of Ku86 and Ku86v to synthetic dsDNA fragments in an EMSA to determine whether Ku86v induces differential DNA-protein complex formation and electrophoretic mobility. We used a 25-mer dsDNA probe for EMSA analysis, because a 25- to 30-bp dsDNA fragment is the minimum length required for the binding of a single Ku heterodimer (8). WCE from two patient MM cells expressing both Ku86 and Ku86v (MM 1 and 2), as well as NBM and CESS cells, were studied. As can be seen in Fig. 2, WCE from CESS, patient MM 1 and 2, as well as NBM formed a complex with the oligomer probe (complex A). EMSA using mAb against Ku86/Ku70 heterodimer (162), Ku86 C terminus (111), and Ku86 N terminus (S10B1) supershifted complex A in all of these samples. Another complex of WCE with the oligomer probe (complex B) was observed only in patients MM 1 and 2. EMSA using S10B1 Ab, but not 162 or 111 Abs, supershifted complex B. These results show that complex B contains Ku86v and suggest that Ku86v might form a Ku86v-DNA complex with differential mobility.

To confirm the formation of this distinct Ku86v-DNA complex in patient MM cells, WCE of patient MM cells with only Ku86v were similarly evaluated in EMSA. As can be seen in Fig. 3, protein-DNA complex formation (complex A) was observed in WCE from three NBM. However, in those patient MM cells expressing only Ku86v (MM 3–14), only Ku86v-DNA (complex B) was observed, confirming the distinct mobility of Ku86v-DNA vs Ku86-DNA complexes. All cell extracts retain DNA binding activity,

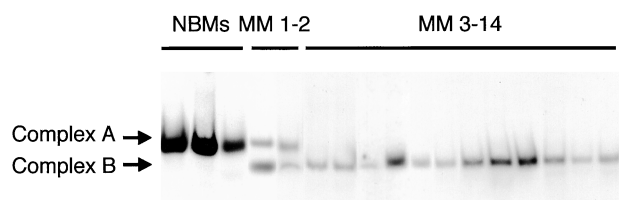


**FIGURE 2.** Differential electrophoretic mobility and DEB activity of Ku86v vs Ku86 in patient MM cells. WCE (1  $\mu$ g) obtained from CESS, patient MM cells with both Ku86 and Ku86v (MM 1 and 2), and NBM were incubated with 25-bp radiolabeled dsDNA probe in the presence of 1  $\mu$ g of closed circular plasmid DNA as a nonspecific competitor. Either no Ab (control) or (1.5–2.0  $\mu$ g) of the following mAbs were preincubated with WCE before adding to binding mixtures: Ab 162 (against Ku86/Ku70 heterodimer); Ab 111 (targets aa 610–705 of Ku86 C terminus); and Ab S10B1 (against aa 8–221 of Ku86 N terminus). The mixtures were then subjected to EMSA analysis on a 5% polyacrylamide gel; the gel was dried and subsequently exposed to x-ray film. Complex A contains Ku86 protein-DNA complexes, and complex B contains Ku86v-DNA complex.

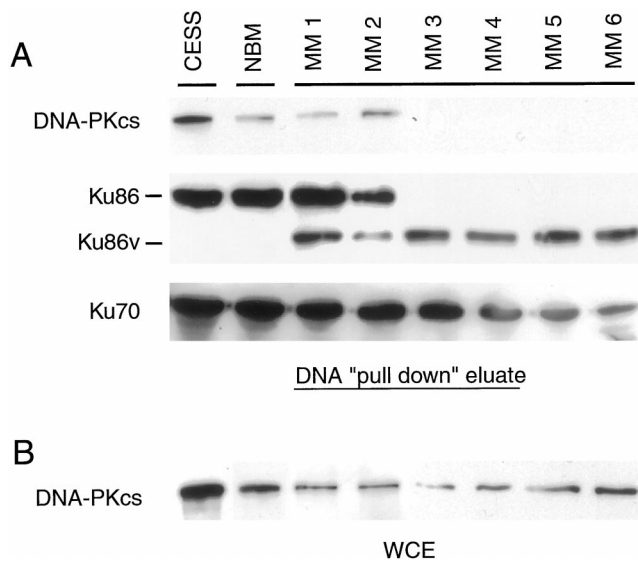
because DNA-protein complexes were readily detectable on EMSA in the absence of either plasmids or poly(dI:dC) as non-specific competitors (data not shown).

#### *MM patient WCE demonstrate decreased Ku-DNA-PKcs complex formation and kinase activity*

After formation of Ku-DNA complexes, DNA-PKcs is recruited and kinase activity is induced. To assay for Ku-DNA-PKcs complex formation and kinase activity, DNA-cellulose was added to WCE from CESS cells, NBM, and patient MM cells, and centrifugation used to “pull-down” DNA-binding proteins, as previously described (18). Proteins associated with DNA cellulose were next eluted and subjected to Western blot analysis. As can be seen in Fig. 4A, Ku86, Ku70 and DNA-PKcs were associated with DNA



**FIGURE 3.** Electrophoretic mobility and DEB activity of Ku86v in patient MM cells. WCE (1  $\mu$ g) obtained from 3 NBM, 2 MM patients with both Ku86 and Ku86v, and 12 MM patients that express only Ku86v (MM 3–14) were incubated with 25-bp radiolabeled dsDNA probe in the presence of 1  $\mu$ g of closed circular plasmid DNA as a nonspecific competitor. Complex A contains Ku86-DNA and complex B contains Ku86v-DNA.



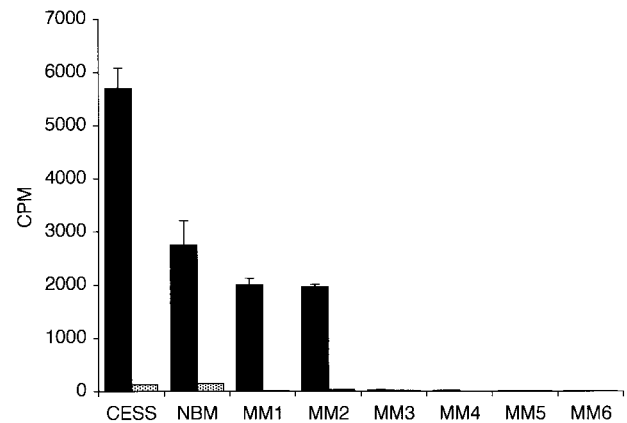
**FIGURE 4.** Ku-DNA-PKcs complex formation is reduced in patient MM cells with Ku86v. DNA-cellulose was added to WCE from CESS cells, NBM, and patient MM cells, and centrifugation used to “pull-down” DNA-binding proteins, as previously described (18). *A*, DNA-binding proteins (150  $\mu$ g) were immunoblotted with anti-DNA-PKcs Ab (18–2), anti-Ku70 Ab (N3H10), and anti-Ku86 Ab (S10B1). *B*, WCE (15  $\mu$ g) from CESS cells, NBM, and patient MM cells were subjected to immunoblotting with anti-DNA-PKcs Ab (18–2).

in NBM and CESS cells. In patients MM 1 and 2, known to express both Ku86 and Ku86v, Ku86, Ku70, and DNA-PKcs were similarly associated with DNA. However, in patients MM 3–6, which express Ku86v but lack Ku86, only Ku70 (but little, if any, DNA-PKcs) was associated with DNA. Patients MM 7–14, which express Ku86v alone, also showed similar association of DNA with Ku70, but not with DNA-PKcs (data not shown). Immunoblotting of WCE from CESS cells, NBM, and patient MM cells confirmed the presence of DNA-PKcs in all samples (Fig. 4*B*). These results demonstrate that the Ku86v-DNA complex in patient MM cells cannot interact with DNA-PKcs.

Given that Ku-DNA-PKcs is required for activation of DNA-PKcs kinase activity, we next determined whether the lack of DNA-PKcs interaction with Ku86v-DNA in patient MM cells correlated with a lack of DNA-PK activity. The DNA cellulose pellets from WCE “pull down” experiments of patient MM cells and NBM were assayed for DNA-PK activity, using wild-type p53 peptide as substrate in the presence of [ $\gamma$ - $^{32}$ P]ATP. To assure specificity of DNA-PK-catalyzed phosphorylation and to control for DNA-PK independent, nonspecific phosphorylation of the p53 peptide, assays were also performed either in the absence of substrate or using mutated p53 peptide as substrate. As shown in Fig. 5, DNA-PK activity was demonstrable in NBM and in CESS cells, as well as in patient MM 1 and 2 cells, but was absent in patients MM 3–6. In patients MM 7–14 that express Ku86v alone, DNA-PK activity was similarly undetectable (data not shown). These results show that DNA-PK activity was detectable only in those MM cells expressing both Ku86 and Ku86v (MM 1 and 2) and is absent in MM cells with only Ku86v (MM 3–6), further confirming the lack of interaction of Ku86v with DNA-PKcs.

#### *Patient MM cells with Ku86v have increased sensitivity to DNA damage*

Because Ku-DNA-PKcs is required for DSB repair and Ku86v-DNA is incapable of forming complexes with DNA-PKcs, we next



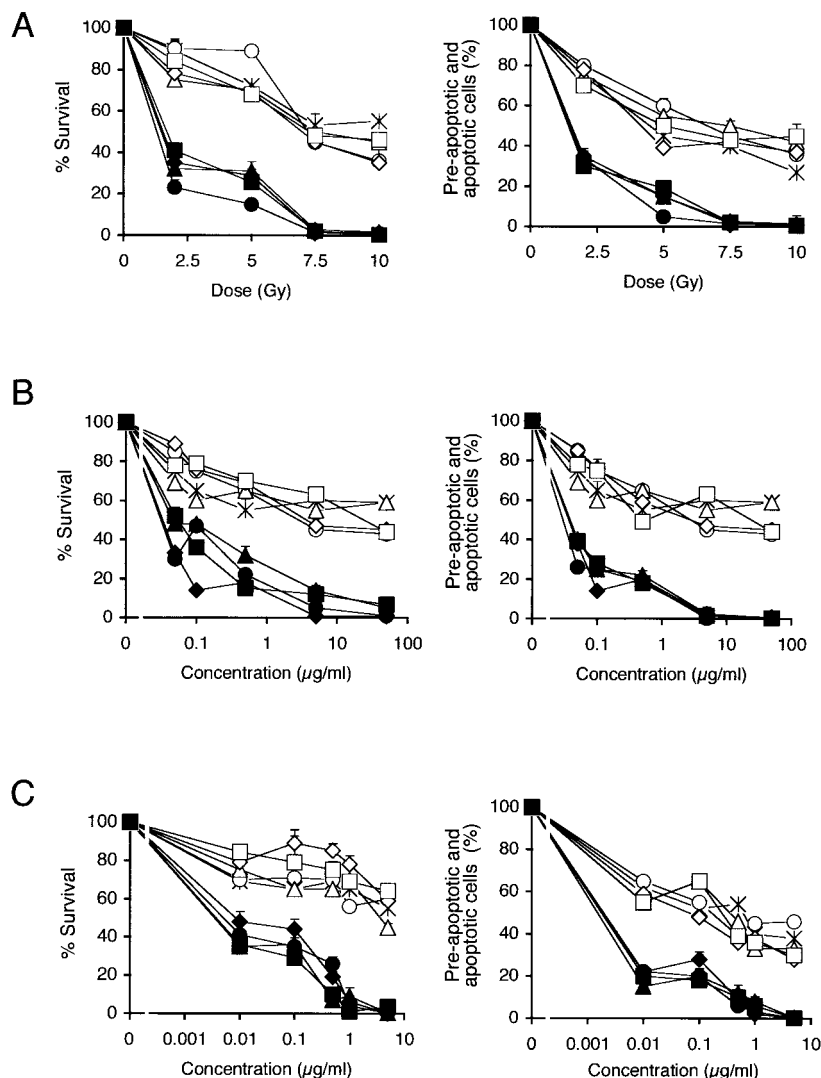
**FIGURE 5.** Ku-DNA-PKcs kinase activity is reduced in patient MM cells with Ku86v. DNA-cellulose was added to WCE from CESS, NBM, and patient MM cells, and centrifugation used to “pull-down” DNA-binding proteins as previously described (18). DNA binding proteins (150  $\mu$ g) were assayed for DNA-PK activity, using wild-type p53 peptide (filled columns) as substrate in the presence of [ $\gamma$ - $^{32}$ P]ATP. To assure specificity of DNA-PK-catalyzed phosphorylation and control for DNA-PK independent, nonspecific phosphorylation of the p53 peptide, assays were also performed either in the absence of substrate or using mutated p53 peptide (dotted columns) as substrate. Values represent mean  $\pm$  SEM of three experiments.

examined the sensitivity of patient MM cells to DNA damaging agents including  $\gamma$  irradiation, mitomycin C, and bleomycin. Specifically, we delineated the number of viable and preapoptotic MM cells, using trypan blue exclusion and annexin V-FITC/FACS analysis, respectively, before and after treatment with these agents. Viability studies using trypan blue exclusion showed that patient MM cells expressing only Ku86v were more sensitive to  $\gamma$  irradiation, mitomycin C, and bleomycin, than patient MM cells expressing both Ku86v and Ku86 or control NBM cells expressing only full-length Ku86 (Fig. 6, *left*). Annexin-V staining also revealed increased apoptotic cells after treatment of Ku86v MM cells vs MM cells expressing both Ku86 and Ku86v or control NBM cells expressing only full-length Ku86 (Fig. 6, *right*).

#### *Ku86 transcripts are detected in patient MM cells with Ku86v*

To determine whether the Ku86v in human MM cells is derived from altered Ku86 transcripts, we performed Northern blot analysis using Ku86 cDNA probes. The 5' and 3' probes for the analysis were obtained by RT-PCR using 5'-1/5'-2 and 3'-1/3'-2 primer pairs in Fig. 8*A*. As shown in Fig. 7, *A* and *B*, Ku86 cDNA probes hybridized to two mRNAs of 2.6 and 3.4 kb in the control NBM sample as previously described in human cell line and normal tissues (35). When 5' probe was used, both Ku86 transcripts were detected in MM samples expressing Ku86v alone (Fig. 7*A*, lanes 4–6). When 3' probe was used, neither band was detected in RNA samples from MM cells expressing Ku86v alone (Fig. 7*B*, lanes 4–6). A  $^{32}$ P end-labeled oligonucleotide probe for 18S rRNA was used as an internal control to ensure the loading and quality of RNA. These results suggest that human MM cells expressed Ku86 transcripts truncated at the 3' end.

Due to the limits in the sensitivity of Northern blotting, we further performed RT-PCR of patient MM samples using primer pairs listed in Fig. 8*A*. These primer pairs allowed the amplification of coding regions of Ku86 cDNA (accession number M30938). RT-PCR for  $\beta$ -actin was served as an internal control for integrity of RNA. RNA from NBM (Fig. 8, *B* and *C*, lane 2)



**FIGURE 6.** Patient MM cells (CD38<sup>+</sup>CD45RA<sup>-</sup>) with Ku86v have increased sensitivity to DNA damage. Patient MM cells expressing either both Ku86 and Ku86v ( $\diamond$ ,  $\square$ ) or Ku86v alone ( $\blacktriangle$ ,  $\blacklozenge$ ,  $\blacksquare$ ,  $\bullet$ ) and the control NBM cells ( $\times$ ,  $\circ$ ,  $\triangle$ ) were treated with  $\gamma$  irradiation (A), mitomycin C (B), or bleomycin (C). Cells were then cultured for 2 days, and the percentage of viable cells in control vs treated cultures was evaluated by trypan blue exclusion (left). The percentage of apoptotic cells was determined by staining with annexin-V and FACS analysis (right). For each dose, the average  $\pm$  SE of triplicates or quadruplicates is given.

was served as a positive control. As shown in Fig. 8B, when primers for 5' Ku86 transcript (5'-1/5'-2) were used, RT-PCR products were detected in all patient MM samples (1097 bp, lanes 3-15). This 5' end product (1097 bp) contains 366 N terminus amino acids of the Ku86 protein from residue 40 to 406. When primers for 3' Ku86 transcript were used (e.g., 3'-1/3'-2), MM samples with both Ku86 and Ku86v (MM 1-2 in lanes 3 and 4) have easily detectable 1082 bp product (Fig. 8C), which is either absent or weakly detected in MM samples with Ku86v alone (MM 3-13 in lanes 5-15). The 3' end product contains 362 C terminus amino acids of the Ku 86 protein from residue 377 to 732, plus 18 bp of untranslated region of Ku transcript. Other 3' Ku86 primer pairs (F2/B2 and F3/B3) similarly amplified detectable RT-PCR products in samples MM 1 and 2, which were either absent or weakly detectable in patients with Ku86v alone (patient MM 3-13) (data not shown).  $\beta$ -actin was readily detected in all patient samples (Fig. 8, B and C). We were able to obtain sufficient purified RT-PCR product for sequencing from the positive samples and confirmed normal Ku86 sequences (36) without mutations or deletions.

#### Sequencing of Ku86v protein

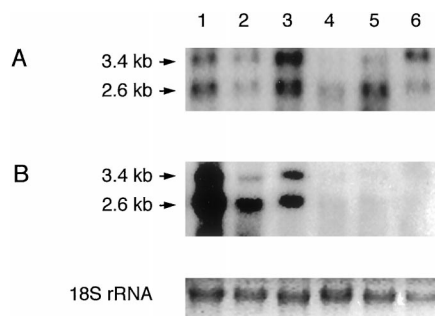
Ku86v protein (69 kDa) was excised from an 8% SDS-PAGE gel and sequencing was performed. Twenty peptides (6-18 amino

acid residues) contained within this 69-kDa protein were sequenced and identical with Ku86.

#### Discussion

In this study, we characterized Ku86 protein and function in freshly isolated patient MM cells. We demonstrated that Ku86v, a 69-kDa variant of Ku86, is expressed in patient MM cells. Immunoblotting using Abs specific for either C or N terminus of Ku86 suggested that Ku86v has truncated C terminus, which was confirmed in EMSA supershift experiments. Ku86v had decreased DEB activity and ability to form complexes with DNA-PKcs, resulting in decreased DNA-PK activity. Ku86v in MM cells correlates with sensitivity to DNA damaging agents and responsiveness to  $\gamma$  irradiation and chemotherapy. In contrast, Ku86 in patient MM cells is associated with resistance to these agents. This study, coupled with reports that increased expression and activity of Ku86 is correlated with chemoresistance, suggests that Ku86 may be a therapeutic target to modulate resistance.

The presence of Ku86v with a truncated C terminus in patient MM cells was suggested by immunoblotting using Abs targeting Ku86 N terminus (S10B1, NC91, and 5E2) and Ab targeting Ku86 C terminus (111), and confirmed using EMSA supershift experiments. To date, the mechanism for generation of Ku86v in MM cells remains undefined and under active investigation. Although

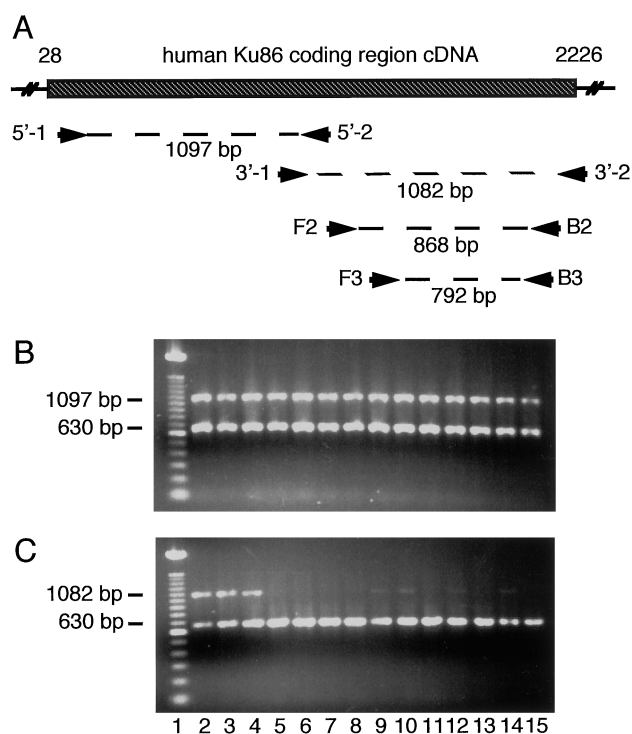


**FIGURE 7.** Patient MM cells (CD38<sup>+</sup>CD45RA<sup>-</sup>) with Ku86v have altered Ku86 transcripts on Northern blot analysis. Five micrograms of total cellular RNA from control NBM (lane 1), patient MM 1–2 (lanes 2 and 3), and patient MM 3–5 (lanes 4–6) were separated on a 1.2% agarose-formaldehyde gel, transferred to nitrocellulose membrane, and hybridized with <sup>32</sup>P-labeled Ku86 cDNA probes: 5' probe (1097 bp) (A) and 3' probe (1082 bp) (B). A <sup>32</sup>P end-labeled oligonucleotide probe for 18S rRNA was used as the loading control. The size of the two Ku86 transcripts detected is indicated in control NBM sample (lane 1).

Ku86v may be derived from spliced variants of Ku86 transcripts, we have not to date observed additional species of Ku86 mRNA on Northern blotting of patient MM cells. In Northern blotting, we have detected normal Ku86 mRNA using 5' probe (Fig. 7A) in all samples; however, no Ku86 transcript (3.4 kb) was detected in those patient MM cells expressing Ku86v alone (patient MM 3–5) using 3' probe (Fig. 7B). Given the limits of detection of Northern blotting, we next used RT-PCR with primer pairs to amplify 5' and 3' Ku86 transcripts. 5' Ku86 transcripts were readily detectable in all patient samples (Fig. 8B), but as shown in Fig. 8C, 3' Ku86 transcripts were either absent or weakly detectable in samples from the majority of patient MM cells expressing Ku86v alone (patient MM 3–13). Specifically, the 5' end product (1097 bp) is present in both normal and all MM cells, whereas the 3' end product (1082 bp) is either absent or weakly detectable in those MM cells with Ku 86v alone. These data are consistent with two possibilities. The first is posttranscriptional modification because the 5' end can be amplified in RT-PCR in all cases, whereas the 3' end cannot be amplified in some samples. The second possibility is that at least some of the MM cells being analyzed are heterozygous at the Ku locus, that wild-type mRNA might be produced from one allele but not both, and that the mutated Ku (Ku86v) has a dominant effect in tumorigenesis.

In these and ongoing studies, the protein lysates were prepared in the presence of proteinase inhibitors, e.g., aprotinin, leupeptin, PMSF, pepstatin, and proteinase inhibitors (proteinase inhibitor cocktail tablets due to reports of an elevated proteolytic activity in human fibroblasts (37). pH was carefully maintained to assure inhibitor activity. We have also performed experiments to lyse MM cells directly in SDS lysing buffer and boiled samples for 5 min immediately before Western blot analysis, a process that inactivates protease activity. Finally, Ku86v (69 kDa) was reproducibly detected in preparations from the majority of MM patients, but not from NBM donors, under identical experimental conditions. The integrity of the protein lysates was assured by immunoblotting for tubulin. In addition, as shown in Fig. 1E, the Ku86v was similarly detected in lysates prepared using SDS-PAGE sample buffer. Therefore, truncated Ku86 protein, as described by Jeng et al. (37), was not present in this study.

Our studies demonstrated that C terminus truncation to form Ku86v has several functional sequelae. First, Ku86v demonstrates distinct electrophoretic mobility than Ku86, with supershifting on EMSA using Ab against Ku86 N terminus, but not with Ab against



**FIGURE 8.** Patient MM cells (CD38<sup>+</sup>CD45RA<sup>-</sup>) with Ku86v have detectable Ku86 transcripts. A, Design and location of primers used in RT-PCR. Shown are the coding regions of Ku86 cDNA (position 28 to 2226). The location of primers are shown by arrow [sense (→) and antisense (←)]. B and C, Agarose gel electrophoresis of RT-PCR products. Shown here are products of RT-PCR using primer pairs 5'-1 and 5'-2 (B), 3'-1 and 3'-2 (C). Duplex PCR were performed for each sample, using a primer pair to amplify  $\beta$ -actin as an internal control (PCR product: 630 bp). An aliquot of the RT-PCR was size fractionated on 0.8% agarose gel and stained with ethidium bromide. 100 bp DNA ladder markers are shown in lane 1; NBM in lane 2; patient MM 1–2 in lanes 3 and 4; and patient MM 3–13 in lanes 5–15.

C terminus. In our hands, Ab against the Ku86/Ku70 heterodimer (38) did not supershift Ku86v in MM cells, suggesting either the lack of heterodimer formation of Ku70 with Ku86v or formation of a Ku70/Ku86v complex that is not recognized by this Ab. Our studies further suggest that Ku70 does form complexes with Ku86v, based upon supershifting of Ku86v EMSA using Ab specific for Ku70. Moreover our "pull down" experiments to characterize DNA binding proteins within patient MM WCE demonstrate the presence of both Ku86v and Ku70. Interestingly, a Ku86 variant with truncated C terminus has been reported in HL-60 promyelocytic leukemia cells and CLL cells that form heterodimers with Ku70 (27–29). This Ku86 variant has molecular mass (~69–71 kDa) similar to the Ku86v in patient MM cells (69 kDa). Therefore, it appears that both Ku86v in HL-60 or CLL cells and Ku86v in patient MM cells can bind Ku70. EMSA demonstrated that Ab against the Ku86/Ku70 heterodimer completely supershifted the DNA-protein complex formed from HL60 cell nuclear extracts (27). However, Ku86v in HL-60 was not sequenced, either at the nucleic acid or peptide level; therefore, we cannot conclude at present whether Ku86v in HL-60 cells is the same Ku86 variant as we have identified in MM cells.

Our studies suggest that Ku86v in patient MM cells has decreased DEB activity compared with Ku86. Numerous yeast hybrid studies have permitted mapping of those regions of Ku86 and Ku70 subunits that bind to each other or to DNA (39–43). A region in the C terminus of both Ku70 and Ku86 (150 aa) appears to be essential for heterodimer formation, whereas larger regions

of both Ku70 and Ku86 are required for effective DEB activity (44). Wang et al. (45) has reported that amino acids 371–510 of Ku86 interact with Ku70, and that amino acids 179–732 of Ku86 (C terminus) are required for DEB. Our results are in accordance with these findings, because the Ku86v in patient MM cells has C terminus truncation and related decreased DEB activity.

Perhaps the most important effect of C terminus truncation on the functional repertoire of Ku86v in patient MM cells is its inability to complex with DNA-PKcs and activate DNA-PKcs, which is essential for repair of DNA damage. Singleton et al. (46) have used a series of C-terminal truncated Ku86 mutants to define the C terminus region required for interaction with DNA-PKcs and kinase activation. This report and the current study both demonstrate that truncation of Ku86 C terminus can result in loss of DNA-PKcs binding and kinase activity. Moreover, in their model using CHO mutants with C terminus deletions (46) as well as in this study of Ku86v in patient MM cells, this lack of Ku86v complex formation with DNA-PKcs and kinase activity resulted in sensitivity to  $\gamma$  irradiation, due to decreased DNA repair. We extended these studies to other DNA damaging agents, namely mitomycin C and bleomycin, and showed that sensitivity of MM cells to these agents correlated with Ku86v expression, and conversely, that expression of Ku86 in patient MM cells conferred relative resistance to these treatments. Therefore these studies shed insight into the mechanism of sensitivity to DNA damage in some MM cells and further suggest that Ku86 may be a potential target to overcome resistance to radiation or chemotherapy. Ongoing studies will examine MM cells freshly isolated from patients both before and after treatment with DNA damaging agents, to correlate Ku86 status with response in vivo, and conversely, to determine whether drug resistant cells have increased expression of normal Ku86. Recent reports have also demonstrated increased Ku86 expression, Ku-DEB activity, and DNA-PK activity both in human chronic lymphocytic leukemia cells resistant to radiation and chemotherapy (47) and in a murine model of leukemia (48). Finally, the recent observation that Ku86-null ES cells exhibit hypersensitivity to chemotherapeutic agents (e.g., etoposide VP-16, bleomycin) (49, 50) further supports the potential utility of targeting Ku86 to modulate chemosensitivity.

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