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Research article

# Effect of Ku proteins on IRES-mediated translation

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*Background information*. Ku is an abundant nuclear heterodimeric protein composed of 70 and 86 kDa subunits. As an activator of the catalytic subunit of DNA-PK (DNA-dependent protein kinase), Ku plays an important role in DNA repair and recombination. Ku is also involved in actions independent of DNA-PK, such as transcription regulation and telomere maintenance. Although Ku is localized in the cytoplasm under specific cellular conditions, no functions for Ku outside of the nucleus have as yet been reported. In addition to DNA binding, Ku binds specific RNA sequences with high affinity. However, no specific cellular mRNA targets for Ku have been identified.

*Results*. In a yeast three-hybrid system, Ku70 bound to an RNA bait that contained an IRES (internal ribosomal entry site) element. A single band with migration properties similar to those of Ku70 was immunoprecipitated with anti-Ku antibody, using UV cross-linked complexes formed by HeLa cell nuclear extracts and an IRES-containing RNA probe. IRES activity was reduced in Ku80$^{-/-}$ cells. Overexpression of Ku proteins stimulated IRES-dependent translation.

*Conclusions*. The present study suggests that Ku binds IRES elements within RNA molecules, and that Ku plays a role in the modulation of IRES-mediated mRNA translation.

## Introduction

Ku is an abundant nuclear protein that binds to DNA double-stranded ends and to other breaks in double-stranded DNA (Dynan and Yoo, 1998). Human Ku is a heterodimer of two subunits of 70 and 86 kDa (designated as Ku70 and Ku80 respectively) that appear to have arisen from a common ancestral gene. After binding to the DNA end, Ku recruits the ~470 kDa DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs) and triggers its kinase activity. The Ku heterodimer and DNA-PKcs (together as the DNA-PK complex) play important roles in the repair of DNA double-stranded breaks induced by ionizing radiation, and in V(D)J recombination, which involves physiological double-strand breakage and rejoining in lymphoid cells (reviewed in Smith and Jackson, 1999). In addition to its association with DNA-PKcs, Ku also has DNA-PK-independent roles, such as transcription regulation and telomere maintenance (Kuhn et al., 1993; Bailey et al., 1999; Samper et al., 2000). In addition to its predominantly nuclear localization, several studies have reported cytoplasmic or cell surface localization of Ku in various cell types (Prabhakar et al., 1990; Dalziel et al., 1992; Fewell and Kuff, 1996; Kumaravel et al., 1998; Koike et al., 1999; Morio et al., 1999), implying that Ku is a multifunctional protein that functions inside, as well as outside, of the nucleus in response to specific signals. The cytoplasmic functions of Ku proteins are still unclear. There is evidence that Ku interacts with RNA, although this interaction has been less well characterized than the interaction with DNA. Using SELEX (systematic evolution of ligand by exponential enrichment) technology, Ku protein was shown to bind with high affinity to specific synthetic RNA sequences (Yoo and Dynan, 1998). In

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**Key words:** internal ribosomal entry site (IRES), Ku, mRNA, RNA binding, translation.

**Abbreviations used:** DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA-PK; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; hnRNP, heterogeneous nuclear ribonucleoprotein; IRES, internal ribosomal entry site(s); MEM, minimum essential medium; PABP, poly(A)$^+$-binding protein; PDGF2, platelet-derived growth factor 2; SELEX, systematic evolution of ligand by exponential enrichment; UTR, untranslated region; VEGF, vascular endothelial growth factor; WT, wild-type.

addition, Ku has been shown to specifically bind to the HIV TAR (transactivation response) element (Kaczmarski and Khan, 1993), to nuclear RNA from HeLa cells (Zhang et al., 2004) and, more recently, to the RNA component of human telomerase (Ting et al., 2005).

A subset of mRNA molecules encoding regulatory proteins, such as growth factors, cytokines, transcription factors and oncogenes, has been found to harbour IRES (internal ribosomal entry site) elements within their extraordinarily cumbersome 5′-UTRs (untranslated regions) (reviewed in Stoneley and Willis, 2004; Komar and Hatzoglou, 2005). Unlike viral IRES elements, which usually lead to efficient translation of the mRNA, cellular IRESs are often weak and highly regulated, allowing them to serve as modulators of translation in response to specific physiological signals. An active IRES promotes the assembly of the translation machinery to secondary/tertiary structures within the 5′-UTR upstream of the initiator AUG codon. The proteins facilitating or inhibiting this recruitment are largely unknown. Cellular IRES elements are thought to confer a translational advantage to cells under conditions of decreased overall rate of protein synthesis, such as during differentiation (Sella et al., 1999), during specific phases of the cell cycle, under various stress conditions and during apoptosis (reviewed in Stoneley and Willis, 2004; Komar and Hatzoglou, 2005).

The present study suggests that Ku70 binds to the 5′-UTR elements of the PDGF2 (platelet-derived growth factor 2) and VEGF (vascular endothelial growth factor) mRNAs, and provides data to propose a role for Ku in modulation of IRES activity.

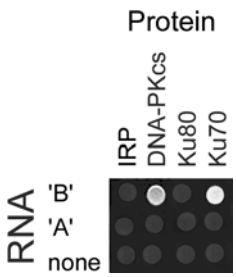
## Results

### Interaction of Ku proteins with IRES RNA

We previously found that a U-rich region spanning nucleotides 475–797 of the PDGF2 5′-UTR (designated region 'B') is important for IRES-mediated translation activity, in contrast with the A-rich region (designated region 'A'), spanning nucleotides 1–227 of the 5′-UTR (Sella et al., 1999). We used a yeast three-hybrid system (Putz et al., 1996) to identify proteins that bind to the IRES and which may regulate its activity. This system for selecting specific RNA–protein interactions in the nucleus of living yeast cells has been successfully applied to the analysis

**Figure 1 | Ku70 promotes the generation of a stable three-hybrid complex with IRES RNA**

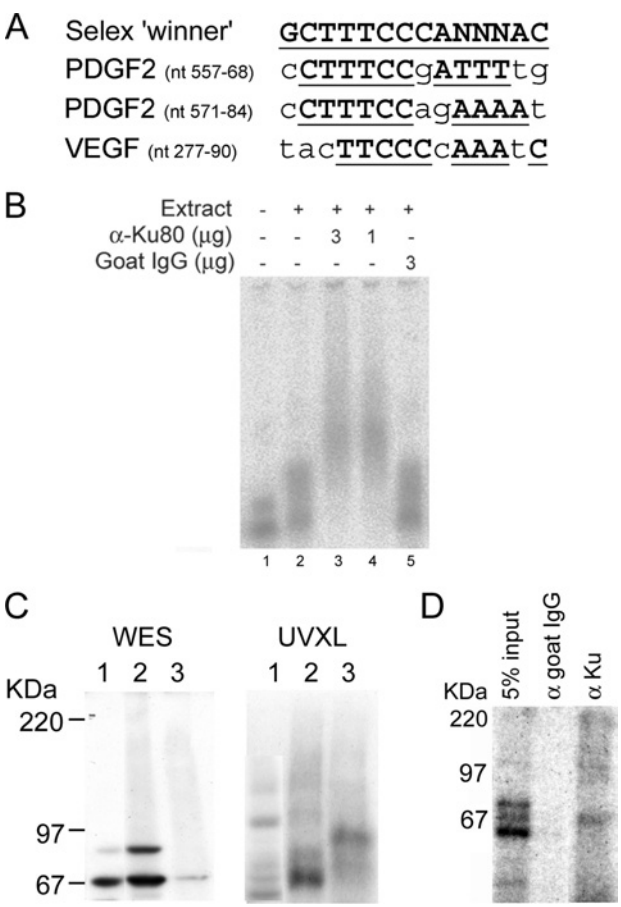
pACTII-IRP, pACTII-DNA-PKcs(3400–3510), pACTII-Ku70 or pACTII-Ku80 expressing the iron-responsive-element-binding protein (IRP), residues 3400–3510 of DNA-PKcs, human Ku70 or human Ku80 respectively were co-transformed with pDBRevM10-'B', pDBRevM10-'A' or pDBRevM10 expressing the various RNA baits (Koloteva-Levine et al., 2002) into yeast. Colonies were selected for HIS3 expression as described in the Materials and methods section.




of the hnRNP-C (heterogeneous nuclear ribonucleoprotein C) interaction with the above-mentioned U-rich region of the PDGF2 IRES (Koloteva-Levine et al., 2002). Using region B as the RNA bait, a human cDNA library derived from HeLa cells (Hannon et al., 1993) was screened for three-hybrid complex formation. Only clones classified as RNA-dependent and RNA-specific (i.e. that could form a stable three-hybrid complex with the RNA bait harbouring region B, but not with region A) were analysed. One of these clones encoded a protein of 110 amino acids, identical with residues 3400–3510 of DNA-PKcs (accession number U34994). Transcription of the *HIS3* reporter gene was activated upon specific interaction of the hybrid RNA containing region B with the DNA-PKcs-(3400–3510) peptide fused to the *GAL4* activation domain (Figure 1). The region of DNA-PKcs used in the present study as a potential RNA-binding protein does not possess any classical RNA-binding motif. Instead, it coincides with the FAT (focal adhesion targeting) domain, also present in the TRRAP subfamily of phosphoinositide kinases, which mediates protein–protein interactions (Bosotti et al., 2000). Interestingly, the binding site for the Ku proteins in the catalytic subunit has been mapped to a region encompassing amino acids 3414–3450 of the catalytic

**Figure 2 | Interaction of Ku70 with IRES RNA**

(**A**) The consensus RNA-binding sequence for Ku aligns with sequences within the IRES elements of PDGF2 and VEGF. The SELEX winner RNA sequence for Ku binding (Yoo and Dynan, 1998) is shown at the top. Alignment with PDGF2 and VEGF 5′-UTRs was performed using the DIALIGN method (Morgenstern, 1999). Bold upper-case letters represent the consensus sequence (top) and the matching nucleotides from PDGF2 and VEGF 5′-UTRs. (**B**) EMSA. Nuclear extracts from K562 cells were incubated with radiolabelled IRES RNA (nt 475–797 of the PDGF2 5′-UTR), and then seperated on a 4% non-denaturing polyacrylamide gel containing 5% glycerol. Lane 1, probe alone; lane 2, probe in the presence of extract; lanes 3, 4 and 5, probe in the presence of extract plus 3 or 1 μg of anti-Ku80 antibody or 3 μg of goat IgG respectively. (**C**) A protein with the mass of Ku70 from a Ku-enriched HeLa extract binds to the IRES RNA. HeLa cell nuclear proteins taken from the Ku-containing fraction (300 mM KCl peak) eluted from a P11 column (lanes 1), or from the further purification of this fraction by passage through a DEAE FF column, followed by step elution with 200 mM or 300 mM KCl (lanes 2 and 3 respectively), were immunobloted with anti-Ku70 antibodies (left-hand panel; WES) or used for a UV cross-linking assay with an IRES RNA probe (nt 475–797 of the PDGF2 5′-UTR) followed by separation by SDS/PAGE (8% gel) (right-hand panel; UVXL). (**D**) Immunoprecipitation of IRES-interacting proteins by anti-Ku antibodies. S7 micrococcal nuclease-treated nuclear extracts (100 μg) from HeLa cells were used for UV cross-linking assay with an IRES RNA probe (nt 1–1054 of VEGF 5′-UTR) (5% input) followed by immunoprecipitation with anti-Ku80 antibody (α Ku) or goat IgG (α goat IgG) and separation by SDS/PAGE (8%).




subunit (Jin et al., 1997). On the basis of the report that Ku function is conserved between yeast and humans (Barnes and Rio, 1997), and the fact that the only known RNA-binding activity for DNA-PK has been mapped to the Ku heterodimer, we assumed that the selection of DNA-PKcs (residues 3400–3510) in the three-hybrid assay was likely to be due to direct binding of yeast Ku with the RNA, leading to a bridging interaction with this region of the catalytic subunit. Use of Ku70 or Ku80 as specific prey in the three-hybrid assay confirmed that Ku70, but not Ku80, binds to the RNA bait (Figure 1). Sequence comparison between the SELEX 'winner' sequence for Ku (Yoo and Dynan, 1998) and the RNA from PDGF2 used in the three-hybrid screen revealed two sites within the PDGF2 IRES that share high sequence similarity to the SELEX winner sequence (Figure 2A). Interestingly, these sequences are located in a stem–loop structure shown to be essential for regulation of IRES function (Sella et al., 1999). In addition, the VEGF IRES element also contains a sequence similar to the SELEX high-affinity consensus sequence for Ku (Figure 2A). To investigate whether Ku binds directly to region B of the PDGF IRES, we performed EMSAs (electrophoretic mobility-shift assays) using a radiolabelled RNA region B and nuclear extracts from differentiated K562 cells [which have been shown previously to support PDGF IRES activity (Sella et al., 1999)] in the presence or absence of an antibody against Ku80. The anti-Ku80 antibody was used in these supershift experiments due to its greater efficiency in immunoprecipitating the Ku70–Ku80 heterodimer. The mobility of the complex formed by the nuclear extracts and the RNA region B probe was decreased by the presence of the anti-Ku antibody, whereas the mobility was not affected by incubation with equal amounts of goat IgG (Figure 2B). Support for the

interaction of Ku with the IRES RNA was obtained by using partially purified Ku proteins from HeLa cells in UV cross-linking experiments. HeLa cells were chosen because of their high Ku content and the ease of growing large amounts for purification. Using a region of the PDGF2 IRES as the probe, we detected an UV cross-linked band corresponding to the size of Ku70 (Figure 2C, right-hand panel, lane 2), which was detected by Western blot analysis of the same fraction, showing that it is enriched for the Ku heterodimer (Figure 2C, left-hand panel, lane 2). To confirm that the cross-linked protein is Ku70, UV cross-linked complexes formed using HeLa cell nuclear extracts were immunoprecipitated by the anti-Ku80 antibody, which specifically immunoprecipitates the Ku heterodimer. Although both Ku70 and Ku80 proteins are brought down using this antibody, a single labelled band with migration properties similar to those of Ku70 was visible (Figure 2D), revealing that Ku70 (but not Ku80) directly interacted with the radiolabelled RNA in the UV cross-linking experiment.

### IRES activity is reduced in Ku80$^{-/-}$ cells

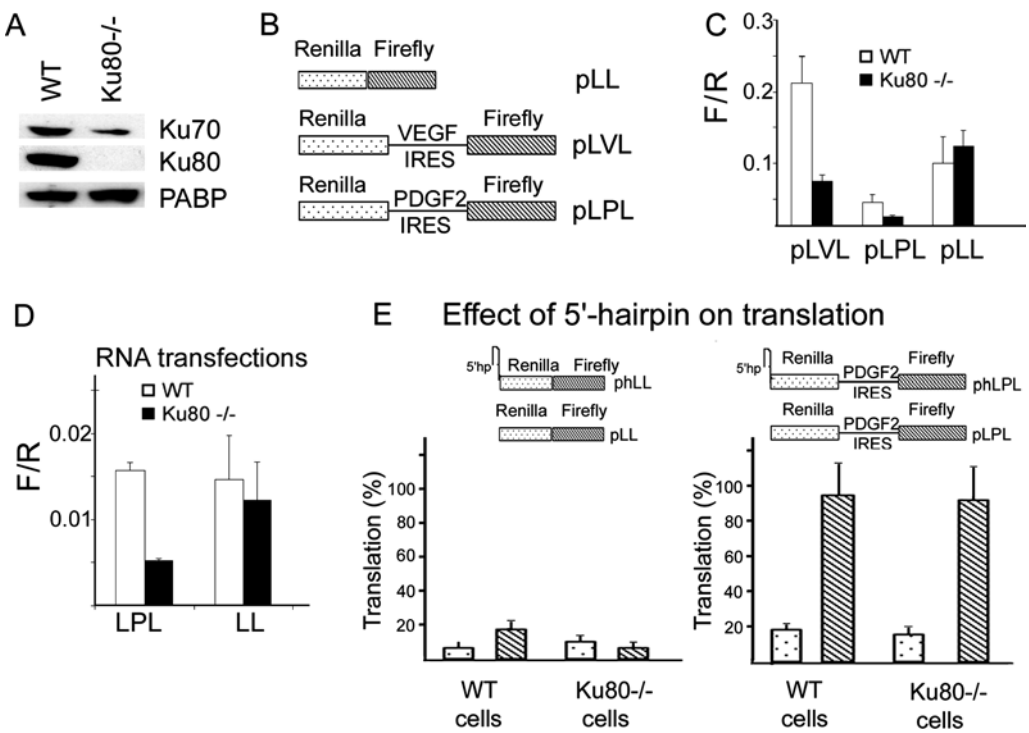
To verify whether Ku plays a functional role in IRES activity, we tested IRES activity in mouse Ku80$^{-/-}$ cells and WT (wild-type) isogenic controls. In Ku80$^{-/-}$ cells the Ku80 subunit is absent and the Ku70 subunit is down-regulated to a low level compared with that in WT cells (Figure 3A), which is in agreement with previous reports (Errami et al., 1996; Singleton et al., 1997). Both cell types were transfected with bi-cistronic reporter plasmids harbouring the IRES elements of PDGF2 or VEGF between the *Renilla* and firefly luciferase coding regions, or with the IRES-minus control plasmid (pLPL, pLVL or pLL respectively; see Figure 3B). Interestingly, there was a 50–60% decrease in the activity of the IRES-mediated second cistron in the Ku80$^{-/-}$ cells compared with WT cells. This was in contrast with translation of the second cistron from the IRES-minus pLL vector (probably through cap-dependent re-initiation) which was similar in both cell types (Figure 3C). To rule out the possibility that a cryptic transcription event was responsible for the higher activity of the IRES-driven reporter gene in the WT cells, *in vitro*-transcribed RNAs encoding the bi-cistronic LPL or LL transcription units were directly transfected into both cell lines. Second cistron translation from a transfected RNA molecule can be due to RNA breakdown, translation read-through or IRES activity. We reason that both RNA breakdown and translation read-through are reflected by the IRES-minus LL mRNA, showing that both are similar in WT and Ku80$^{-/-}$ cells (Figure 3D, LL). However, translation of the second cistron from the IRES-containing LPL mRNA was significantly lower in the Ku80$^{-/-}$ cells, raising the possibility that the IRES activity is less efficient in these cells compared with WT cells (Figure 3D, LPL). To further demonstrate that the translation of the second cistron from the IRES-containing plasmid is 5′-end independent, we included a second set of plasmids that encode a stable 5′-hairpin upstream of the *Renilla* sequence (Figure 3E, phLL and phLPL) in order to inhibit the 5′-cap-dependent translation of the first cistron. In this experiment, we compared the effect of the 5′-hairpin on translation of both cistrons in both cell types. As expected, the 5′-hairpin significantly reduced the translation of the first cistron from the IRES-containing (pLPL) and the IRES-minus (pLL) plasmids, as well as the translation of the second cistron from the IRES-minus plasmid. However, the 5′-hairpin did not have an inhibitory effect on the IRES-mediated translation of the second cistron (pLPL). This experiment confirms the activity of the IRES element in both WT and Ku80$^{-/-}$ cells (Figure 3E), regardless of the fact that its activity in Ku80$^{-/-}$ cells is significantly lower compared with WT cells (Figures 3C and 3D).

### Overexpression of Ku proteins stimulates IRES-dependent translation

To verify if the defect in IRES-dependent translation in the Ku80$^{-/-}$ cells was due to the absence of Ku80 or to the reduction in the amount of Ku70 in these cells, we performed overexpression analysis. WT and Ku80$^{-/-}$ cells were transfected with increasing amounts of Ku80- or Ku70-expressing plasmids. The total amount of the transfected Ku80 in Ku80$^{-/-}$ cells was below the detection level of the anti-Ku80 antibody on the Western blot, but was evident by the increase in the stability of endogenous Ku70 (Figure 4A), consistent with previous results (Singleton et al., 1997). These increases in endogenous steady-state levels of Ku70 as a result of Ku80 over-expression were mirrored in WT cells, where the increase in Ku80 levels was readily detectable

**Figure 3 | IRES activity is decreased in Ku80$^{-/-}$ cell lines**

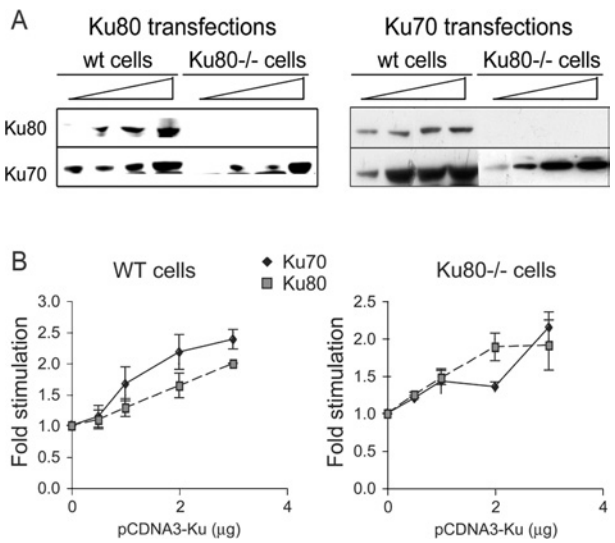
(**A**) Steady-state levels of Ku70 are decreased in cells lacking Ku80. Nuclear extracts (20 μg of protein) from WT or Ku80$^{-/-}$ cells were separated by SDS/PAGE (8% gel) and immunobloted with anti-Ku70, anti-Ku80 or anti-PABP antibodies as indicated. (**B**) Schematic representation of transfected bi-cistronic plasmids. The bi-cistronic plasmids express *Renilla* and firefly luciferase reporter genes from the first and second cistrons respectively, under the control of CMV (cytomegalovirus) promoter (Gerlitz et al., 2002). The IRES elements of VEGF or PDGF2 are in the intercistronic space of pLVL or pLPL respectively. pLL is the bi-cistronic IRES-minus control plasmid. (**C**) IRES-mediated translation is reduced in Ku80$^{-/-}$ cells. WT or Ku80$^{-/-}$ cells were transfected with the bi-cistronic plasmids pLL, pLVL or pLPL. After transfection (48 h), the cells were assayed for *Renilla* (R) and firefly (F) luciferase activities. F/R values obtained for WT and Ku80$^{-/-}$ cells are shown. Results are the means ± S.E.M. for at least 5 independent experiments. (**D**) Reduction of IRES activity in Ku80$^{-/-}$ cells is not due to transcription from a cryptic promoter. WT or Ku80$^{-/-}$ cells were transfected with bi-cistronic mRNAs that were transcribed *in vitro* using pLPL or pLL as templates. After transfection (24 h), the cells were assayed for *Renilla* (R) and firefly (F) luciferases activities. F/R values obtained for WT and Ku80$^{-/-}$ cells are shown. Results are the means ± S.E.M. for 3 independent experiments. (**E**) Effect of 5′-hairpin (5′ hp) on translation in WT and Ku80$^{-/-}$ cells. pLL or pLPL (or those containing 5′-hairpin upstream of the *Renilla* cistron, i.e. phLL and phLPL) were transfected into WT or Ku80$^{-/-}$ cells. After transfection (48 h), the cells were assayed for luciferase activities. *Renilla* (R) or firefly (F) luciferase units obtained from pLL or pLPL lacking the 5′-hairpin were set as 100%. R or F luciferase units obtained from phLL or phLPL containing the 5′-hairpin were compared with the R or F units obtained from the pLL or pLPL lacking the 5′-hairpin. The results, representing the effect of the 5′-hairpin on translation from the first and second cistrons in WT or Ku80$^{-/-}$ cells, are the means ± S.E.M. for 3 independent experiments.




(Figure 4A), confirming that the plasmid correctly expressed Ku80. Similarly, Ku70 overexpression led to the stabilization of Ku80 in WT cells (Figure 4A). To test the effect of transfected Ku proteins on IRES activity, the bi-cistronic plasmid pLPL was co-transfected with increasing amounts of Ku80- or Ku70-expressing plasmids. IRES-dependent activity was stimulated in a dose-dependent manner by the expression of either Ku70 or Ku80 in the Ku80$^{-/-}$ cells, as well as in the WT cells (Figure 4B). Since the overexpression of Ku80 in both cell lines was accompanied by the stabilization of Ku70, and the overexpression of Ku70 alone in the Ku80$^{-/-}$ cells led to similar levels of stimulation of IRES-dependent

**Figure 4 | Effect of Ku80 and Ku70 overexpression on IRES activity**

(**A**) Overexpression of Ku80 and Ku70 in WT and Ku80$^{-/-}$ cells. WT and Ku80$^{-/-}$ cells were transfected with 0, 0.5, 2 or 4 μg of pcDNA3 expressing Ku80 or Ku70. After transfection (48 h), 50 μg of protein of whole-cell extracts was separated by SDS/PAGE (8% gel) followed by immunodetection using antibodies specific for Ku70 and Ku80. (**B**) Overexpression of Ku80 and Ku70 stimulates IRES activity in WT and Ku80$^{-/-}$ cells. Cells were transfected with 1.5 μg of pLPL (see Figure 3B), 0–4 μg of pcDNA-Ku80 and 0–4 μg of pcDNA3-CAT to make the total amount of transfected DNA to 5.5 μg. After transfection (48 h), the cells were harvested and assayed for *Renilla* (R) and firefly (F) luciferase activities. Results are expressed as the fold stimulation of F/R activity in Ku70- or Ku80-transfected cells over the F/R ratio in GFP (green fluorescent protein)-transfected cells, and are expressed as the means ± S.E.M. for at least 3 independent experiments.




translation as in the WT cells, these results suggest that Ku70 could act independently of Ku80 to modulate IRES activity.

## Discussion

Ku has been previously shown to have nuclear DNA-PKcs-independent functions in both transcriptional regulation and telomere maintenance (Kuhn et al., 1993; Bailey et al., 1999; Samper et al., 2000). The DNA-PKcs-independent roles of Ku are evident from Ku70- or Ku80-knockout mice, which exhibit defects in cell cycle and growth control that are not observed in DNA-PKcs-knockout mice (Nussenzweig et al., 1996; Gao et al., 1998; Gu et al., 2000). Several previous reports, using Ku70–Ku80 heterodimers purified from cells, have demonstrated the ability of Ku to form complexes with RNA (Reeves, 1985; Kaczmarski and Khan, 1993; Yoo and Dynan, 1998; Zhang et al., 2004). The present study suggests that Ku70 independently interacts with the RNA, but extensive biochemical work with purified recombinant monomers, in addition to the heterodimer, is required to elucidate if Ku70 binding to RNA is purely Ku80-independent when both subunits of the heterodimer are present. Previously, Ku70 has been shown to function in the cytosoplasm as a suppressor of the apoptotic translocation of Bax to the mitochondria (Sawada et al., 2003), supporting the idea that it may have an additional Ku80-independent role.

In the present study we demonstrate that Ku70 interacts with an IRES-bearing RNA molecule which contains a sequence almost identical to that identified by the SELEX technique as the winner sequence for Ku binding (Yoo and Dynan, 1998). In addition, an antibody that specifically recognizes the Ku heterodimer supershifts the RNA–protein complex (Figure 2). Moreover, a three-hybrid assay shows an interaction between the IRES RNA and Ku70 (Figure 1). In addition to the interaction with the IRES RNAs, the Ku heterodimer appears to be modulating their translational activities (Figure 4). It is still unclear whether both Ku80 and Ku70 are involved in this role, or whether Ku70 is acting alone to modulate translation. In support of the notion that Ku70 acts independently of Ku80 is the stimulation of IRES activity in Ku80$^{-/-}$ cells by Ku70 overexpression (Figure 4B). At this point we cannot rule out the possibility that Ku proteins could affect IRES-mediated translation indirectly, such as by regulating the transcription of another modulator of the IRES. Extensive mutagenesis analysis of the putative Ku-binding site within the IRES RNA, followed by binding assays and IRES function assays, will provide the final proof for the direct role of Ku in modulation of IRES-mediated translation.

Recently, in addition to the roles in DNA repair and telomere maintanance described for Ku, it was reported that the DNA–PK complex phosphorylates several hnRNPs in an RNA-dependent manner (Zhang et al., 2004). The set of proteins described include hnRNP-C, a protein previously described by our laboratory (Sella et al., 1999) and by others

(Holcik et al., 2003; Kim et al., 2003) to be involved in the regulation of IRES-mediated translation. Since PDGF2 IRES activation correlates with the hyperphosphorylation of hnRNP-C (Sella et al., 1999), an intriguing possibility is the involvement of Ku as a sensor of specific signals, leading to hnRNP-C phosphorylation and IRES activation. IRES-containing mRNA molecules are subjected to stringent translational regulation, as expected for a subclass of genes encoding regulatory proteins that govern cell growth, cell cycle, differentiation and apoptosis. Cellular IRES elements have been implicated in conferring a translational advantage in response to specific physiological signals or under stress conditions. Future experiments will reveal the mechanism by which Ku modulates the translation efficiency of specific mRNAs.

## Materials and methods

### Plasmids

To PCR amplify the Ku80 or Ku70 cDNAs respectively from human B-cells the following oligonucleotide primer pairs were used: 5′-CGCGGATCCAAATGGTGCGGTCG-3′ and 5′-CGCCTCGAGTTACTATATCATGTCCAATAAA-3′; 5′-CGCGGATCCAAATGTCAGGGTGG-3′ and 5′-CGCCTCGAGTTATCAGTCCTGGAAGTGC-3′. The PCR products were digested with BamHI and XhoI (Roche) and ligated into the BamHI–XhoI sites of pCDNA3 vector (Invitrogen) to create pCDNA3-Ku80 and pCDNA-Ku70, or into BamHI–XhoI sites of pACTII (Clontech) to generate pACTII-Ku80 and pACII-Ku70.

The bi-cistronic plasmids pLL, pLPL and pLVL have been described previously (Gerlitz et al., 2002). To create the 5′-hairpin versions of these bi-cistronic plasmids we used the oligonucleotide primers 5′-TTCCAAAAGAAGTCGAGTGGG-3′ (3′ of the hairpin) and 5′-ATGTGCTGCAAGGCGATTAAG-3′ (5′ of the hairpin) to amplify a 100-nt fragment using pBS-hpGST (Professor B. Levy, Technion, Haifa, Israel) as a template. The PCR products were blunt-ended and ligated into the blunt-ended SalI site of pCMV-LL and pCMV-LPL to generate pCMV-hLL and pCMV-hLPL respectively.

### Three-hybrid screen

The technique described by Putz et al. (1996) was employed for three-hybrid screen and analyses. For the screen, 1000 μg of cDNA library from human HeLa cells fused to the GAL4-activation domain (Hannon et al., 1993) was used to co-transfect the yeast strain CG1945 (Clontech), together with 500 μg of pDBRevM10-'B' (Koloteva-Levine et al., 2002). Transformants ($5\times10^5$) were allowed to grow for 10 days at 30°C on $His^-$ plates supplemented with 0.5 mM 3-aminotriazole (Sigma). Plasmids were rescued from each of the 230 surviving colonies and used for re-transformation, together with either pDBRevM10-'A' or pDBRevM10 (Koloteva-Levine et al., 2002) which have a different or no RNA bait respectively. Only clones that exhibited RNA-dependent and RNA-specific growth were characterized further. Double transformants forming a stable three-hybrid association were selected for HIS3 expression by adding 10 μl of a liquid culture, grown to $D_{600}$ of 0.1, on to plates with 0.5 mM 3-aminotriazole and lacking tryptophan, leucine and histidine. Cells were allowed to grow for 5 days at 30°C.

### Cells, transfections and luciferase assays

HeLa S3 suspension cells were grown in MEM (minimum essential medium) supplemented with 10% newborn calf serum, 1% penicillin/streptomycin and 1% L-glutamine. $Ku80^{-/-}$ and isogenic WT mouse cell lines (Nussenzweig et al., 1996; Peterson et al., 1997) were grown in α-MEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin/kanamycin (Biological Industries). Cells grown in 12-well plates were transfected with 2 μg or the indicated amounts of plasmid using Superfect transfection reagent (Qiagen). Cells were harvested 48 h post-transfection and assayed for *Renilla* and firefly luciferase activities using the dual-luciferase reporter assay system (Promega) and a TD-20e-Luminometer (Turner).

### RNA transfections

Cells were transfected with *in vitro*-transcribed mRNAs (Amplicap T7 high yield transcription kit; Epicentre Technologies) using the Transmessenger RNA transfection reagent (Qiagen), as recommended by the manufacturer, and harvested 24 h post-transfection for dual-luciferase analysis.

### Preparation of cell extracts and immunodetection

Whole-cell extracts were prepared using buffer containing 25 mM KOH/Hepes (pH 7.5), 1% Triton X-100 and 100 mM KCl with freshly added 1 mM DTT (dithiothreitol) and protease inhibitor cocktail (Complete™; Roche). Nuclear and cytoplasmic extracts were prepared as described previously (Abmayr and Workman, 1998). For treatment of nuclear extracts with S7 micrococcal nuclease, extracts were incubated with 1 mM $CaCl_2$ and 75 units/ml of S7 nuclease (Roche) for 20 min at room temperature followed by the addition of 2 mM EGTA. Western-blot analyses were performed according to standard procedures using anti-Ku70 (Serotec), anti-Ku80 (Santa-Cruz Biotech), anti-PABP [poly(A)$^+$-binding protein] and anti-hnRNP-C antibodies (gifts from Professor G. Dreyfuss, University of Pennsylvania School of Medicine, Philadelphia, U.S.A.). Immunoprecipitations were carried out using anti-Ku80 antibody after incubation overnight at 4°C in PBS containing 0.1% Nonidet P40.

### Partial purification of Ku proteins

Partial purification of Ku proteins from nuclei of HeLa S3 cells was performed as described previously (Dvir et al., 1993). Ku proteins were eluted from the P11 column (Pharmacia) with 300 mM KCl (as estimated by Western blot analysis). Positive fractions were pooled and diluted to a concentration of 100 mM KCl before being passed through a DEAE FF column (Pharmacia). The DEAE column was eluted with steps of 200 mM and 300 mM KCl.

### EMSA

The probe for EMSA (spanning nt 475–685 of the PDGF2 5′-UTR) was uniformly labelled by incorporation of [α-$^{32}$P]UTP during *in vitro* T7 polymerase transcription. The binding reactions (in a final volume of 20 μl) were carried out in buffer

containing 5 mM Hepes, pH 7.9, 40 mM KCl, 2 mM $MgCl_2$, 4% glycerol, 2 mM DTT, 1 mg/ml *Saccharomyces cerevisiae* tRNA and 3 ng of uniformly labelled RNA (35 000 c.p.m.). K562 nuclear extracts were obtained as described previously (Sella et al., 1999). Reactions were incubated for 10 min and loaded on to 4% non-denaturing polyacrylamide gels containing 5% glycerol.

### UV cross-linking assay

Uniformly radiolabelled RNA probes (5 fmol; spanning nt 475–685 of the PDGF2 5′-UTR or nucleotides 1–1054 of the VEGF 5′-UTR), as described previously (Sella et al., 1999), were incubated with the indicated cell extracts in a final volume of 20 μl containing 10 mM Hepes (pH 7.6), 30 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 2 mM DTT and 1 mg/ml *Saccharomyces cerevisiae* tRNA for 10 min at 37°C, followed by exposure to 312 nm radiation for 15 min using an Ultralum UVC-508 UV cross-linker. Reactions were treated by the addition of 10 μg of RNaseA for 1 h at 37°C. The resulting complexes were resolved by SDS/PAGE (10% gel) followed by autoradiography or exposure to a phosphorimager screen.

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