KARP-1: a novel leucine zipper protein expressed from the Ku86 autoantigen locus is implicated in the control of DNA-dependent protein kinase activity

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The Ku autoantigen plays an integral role in mammalian DNA double-strand break repair as the DNA binding component of the DNA-dependent protein kinase (DNA-PK) complex. Here, we demonstrate that a second gene, KARP-1 (Ku86 Autoantigen Related Protein-1), is expressed from the Ku86 locus. The KARP-1 gene utilizes an upstream promoter and additional exons which results in an extra 9 kDa of protein appended onto the normal Ku86 polypeptide. The KARP-1-specific domain encodes interdigitating hexa- and penta-heptad repeats of leucine residues flanked by a very basic region. Intriguingly, the catalytic subunit of DNA-PK also contains a hexa-heptad repeat of leucines. Consistent with this observation, we observed that human cell lines stably expressing dominant-negative constructs of KARP-1 resulted in diminished DNA-PK activity and X-ray hypersensitivity and that a KARP-1 antibody significantly neutralized DNA-PK activity *in vitro*. Finally, we present data which suggests that KARP-1 may be primate-specific. These observations have important repercussions for mammalian DNA double-strand break repair.

Keywords: DNA-PK/DNA repair/Ku86/leucine zipper

Introduction

Ku protein was originally identified as an autoantigen recognized by sera from patients with rheumatic disorders (Mimori *et al.*, 1981). It was subsequently found to be an autoantigen in patients with several other autoimmune diseases, including systemic lupus erythematosus, Graves' disease and Sjogren's syndrome (Mimori *et al.*, 1981; Reeves, 1985). Ku antigen was purified by immunoaffinity and shown to be a heterodimer consisting of 86 kDa (Ku86) and 70 kDa (Ku70) subunits (Reeves, 1985; Yaneva *et al.*, 1985; Francoeur *et al.*, 1986; Mimori *et al.*, 1986). Biochemical analyses of Ku demonstrated that it bound in a sequence non-specific fashion to virtually all double-stranded DNA ends including 5'- or 3'-protruding ends, blunt ends (Mimori and Hardin, 1986), and duplex DNA ending in stem-loop structures (Falzon *et al.*, 1993), apparently by recognizing transitions from double- to single-stranded DNA. Ku has also been reported to be capable of sequence-specific binding, particularly within

the promoter elements of genes (Giffin *et al.*, 1996). However, since at least six completely different sequence-specific binding sites have been described for Ku (Knuth *et al.*, 1990; Liu and Lee, 1991; Hoff and Jacob, 1993; Beall *et al.*, 1994; Genersch *et al.*, 1995; Giffin *et al.*, 1996), the biological relevance of these studies is currently unclear. Ku has also been shown to possess DNA-dependent ATPase (Cao *et al.*, 1994) and helicase activities (Tuteja *et al.*, 1994). Lastly, Ku is biochemically and genetically well-conserved, with homologs having been described in monkeys (Paillard and Strauss, 1991), rodents (Mimori *et al.*, 1990; Porges *et al.*, 1990; Errami *et al.*, 1996; He *et al.*, 1996; Lee *et al.*, 1996), *Drosophila melanogaster* (Jacoby and Wensink, 1994), and *Saccharomyces cerevisiae* (Feldmann and Winnacker, 1993; Boulton and Jackson, 1996; Milne *et al.*, 1996). Due to its conservation and to the multitude of activities ascribed to it, Ku has been postulated to participate in recombination, repair, replication and/or transcription (reviewed in Jeggo *et al.*, 1995; Anderson and Carter, 1996).

One unequivocal role for Ku is as a DNA-binding subunit of the DNA-dependent protein kinase (DNA-PK) complex. DNA-PK is a serine-threonine protein kinase that requires the presence of double-stranded DNA with free ends for its activity and is composed of the Ku heterodimer and a 465 kDa catalytic subunit (DNA-PK_{cs}) (Dvir *et al.*, 1992; Gottlieb and Jackson, 1993; Suwa *et al.*, 1994). Recently, extensive genetic and molecular analyses have identified DNA-PK as an integral component of the DNA double-strand break (DSB) repair pathway (reviewed in Jeggo *et al.*, 1995; Jackson, 1996).

In mammals, defects in DNA DSB repair manifest themselves in two easily recognizable phenotypes: ionizing radiation (IR) hypersensitivity and immunodeficiency. These two seemingly unrelated biological processes are in fact linked by the requirement of DNA DSBs as reaction intermediates. Thus, the exposure of mammalian cells to IR induces lesions in chromosomal DNA such as strand scissions, single-stranded breaks, DSBs and base cross-links (Price, 1993). In particular, DNA DSBs appear to be the predominant cytotoxic lesions as even a single unrepaired DNA DSB can be a lethal event (Klar *et al.*, 1984; Frankenberg-Schwager and Frankenberg, 1990). Mammalian IR-sensitive (IR^s) mutants have been isolated and in approximately half of these cell lines, IR sensitivity correlated with a greatly decreased ability to repair DNA DSBs (reviewed in Zdzienicka, 1995). Thus, the DSB repair capacity of a cell appears to be a critical, though not the sole, factor in determining cellular IR-sensitivity. Similarly, the development of the mammalian immune system is dependent upon a site-specific DNA recombination process, termed lymphoid V(D)J recombination, that assembles the non-contiguous genomic segments that encode the Variable (V), Diversity (D), and Joining (J)

elements of immunoglobulin and T-cell receptor genes (reviewed in Lewis, 1994). Importantly, analyses of V(D)J recombination products *in vivo* and *in vitro* has proven that DNA DSBs are an essential intermediate in the V(D)J reaction mechanism (reviewed in Oettinger, 1996). Thus, the repair of DNA DSBs is an integral feature of IR sensitivity and V(D)J recombination.

Consistent with this hypothesis, mutations in the subunits of DNA-PK have been shown to affect deleteriously both IR sensitivity and V(D)J recombination. DNA-PK_{cs} is now known to be the product of the severe combined immune deficiency (*scid*) gene (Blunt *et al.*, 1995; Hartley *et al.*, 1995; Kirchgessner *et al.*, 1995; Lees-Miller *et al.*, 1995; Danska *et al.*, 1996) and it has long been recognized that animals homozygously defective at this locus were profoundly immune deficient (Bosma *et al.*, 1983), IR^s (Fulop and Phillips, 1990) and defective in DNA DSB repair (Biedermann *et al.*, 1991; Hendrickson *et al.*, 1991). Recently, it was shown that cell lines belonging to the fifth X-ray cross-complementation group (XRCC5) (Thompson and Jeggo, 1995; Zdzienicka, 1995), which were known to be IR^s and V(D)J-defective, were deficient in Ku86 gene expression (Smider *et al.*, 1994; Taccioli *et al.*, 1994; Boubnov *et al.*, 1995; Errami *et al.*, 1996; He *et al.*, 1996). Lastly, knock-out mice for Ku86 have been generated by homologous recombination (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996) and, as expected, these mice have a profound immune deficiency and are IR^s. Thus, DNA-PK has been unequivocally identified as an important mammalian DNA repair complex and mutations in either DNA-PK_{cs} or the 86 kDa subunit of Ku result in severe IR^s and V(D)J recombination deficits due to impaired DNA DSB repair.

In the course of analyzing the putative promoter region for human Ku86 we serendipitously discovered that a second gene, termed KARP-1 (Ku86 Autoantigen Related Protein-1), can be encoded by the Ku86 locus. KARP-1 is expressed using an alternative promoter and splicing and produces a protein which consists of ~9 kDa of additional polypeptide appended onto the N-terminus of Ku86. The KARP-1-specific domain encodes a novel leucine repeat motif and a very basic region. Stable human cell lines expressing dominant-negative versions of KARP-1 resulted in diminished DNA-PK activity and IR^s. These observations have important implications for DNA-PK activity and mammalian DNA DSB repair.

Results

Identification of a novel open reading frame in the human Ku86 promoter region

The presumptive promoter region of the human Ku86 gene was obtained by PCR using primers derived from the 5'-end of the human Ku86 sequence (Mimori *et al.*, 1990) and a commercially available genomic DNA isolation kit (GenomeWalker, Clontech). Initially, a 1.2 kb DNA fragment was isolated and sequenced. This information was utilized to isolate by PCR an additional fragment. Together, these fragments comprised ~4 kb of genomic DNA sequence upstream of the initiator ATG for the Ku86 gene. Subsequently, much of this sequence was verified by sequencing the corresponding regions from clones, isolated by conventional colony hybridization screening, of

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1      ttttaagtaaatctgtctgtactagCGTTTCAGGCGGCTCAAAACACACAGCCCCGACTA  60
61      CGGCGGAATGGAGAGAATGTGCGCATCTCGCGGGAATCTGCGCATGCTCGGAGAGAAT  120
           M L G G N L R M L G E N
           *
121      CTGCGCATGCTCGGCGGAATCTGCGGAGCTCGCGGGAATCTGCGCAAGCTCGCGGG  180
           L R M L G R N L R E L G G N L R K L G G
           *
181      AATCTGCGCATGCTCAGAGTTCGCGGACGCGTTTCGCCGCCCTTCAGGCTAGCAGG  240
           N L R M L R V P G H G F P A P F R P S R
           *
241      AAACGAAGCGGCTCTTTCCGCTATCTGCGCGCTTGTCACCGGAAGAGAGTTCGACACGG  300
           K R S G S F R Y L P L V H R K R V A T R
           + +
301      CAGGTTCCCGCCGGAAGCGACAGAGCGCTGAGGACCGGCAACATGGTGGCGTGG  360
           Q V P A R K K R P E R L R T G N M V R S
           + + + + +
           Ku86>

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Fig. 1. The KARP-1 open reading frame (ORF) exists in the genomic DNA upstream of the human Ku86 gene. Lower-case letters correspond to intronic DNA, whereas upper-case letters correspond to KARP-1 exon DNA and the bold, upper-case letters correspond to the Ku86 gene. The predicted KARP-1 protein sequence is shown using the single letter amino acid code. The leucine residues in the hexa-heptad repeat are shown by a bold asterisk (*) while the leucine residues in the penta-heptad repeat are shown by a normal asterisk (*). Basic amino acids in the flanking domain are marked with a plus (+). A putative nuclear localization signal is shown by the horizontal line.

an independent human genomic library. Very unexpectedly, we identified within this region an open reading frame (ORF) upstream of Ku86 that was unusual for four reasons (Figure 1). First, the ORF was in-frame and contiguous with Ku86 and extended at least an additional 88 amino acids beyond the Ku86 ATG (Figure 1), whereas stop codons are usually found upstream of the initiator ATG for most mammalian genes (Kozak, 1987). Secondly, in the few cases where long ORFs in the 5'-untranslated region (UTR) of genes have been described they usually do not encode additional potential initiator methionine residues (Kozak, 1991; Xiong *et al.*, 1991). The Ku86 5'-UTR ORF, however, encoded several in-frame methionines (Figure 1). Thirdly, the Ku86 5'-UTR ORF contained an extremely basic region in which 15 of 37 residues were either lysines or arginines, including a potential nuclear localization signal (Kalderon *et al.*, 1984). Lastly, the Ku86 5'-UTR ORF also had the capacity to encode a novel leucine repeat which consisted of a perfect hexa-heptad repeat of leucine residues interdigitated with a perfect penta-heptad repeat of leucines (Figures 1 and 2A). Heptad repeats of leucines are characteristic of a subset of coiled-coil proteins known as leucine zippers. These proteins have a characteristic seven-residue repeat, (a-b-c-d-e-f-g)_n in which a leucine residue is found at all or most of the 'd' positions and hydrophobic residues are found at the 'a' positions (Landschulz *et al.*, 1988; reviewed in Hurst, 1994). The leucine repeats observed in the Ku86 5'-UTR ORF conformed to this pattern (Figure 2A).

Analysis of the ORF sequence revealed two additional features. First, a computer search suggested that the leucine repeat sequence had weak homology (20% identity and 40% similarity) to the Hin integrase (Figure 2B). Recently, it has been shown that the RAG-1 gene (Schatz *et al.*, 1989), which is absolutely required for V(D)J recombination (Mombaerts *et al.*, 1992) also has functional homology to the Hin integrase and that the recombination signal sequences which mediate V(D)J recombination resemble the Hin recombination site (Spanopoulou *et al.*, 1996; reviewed by Lewis and Wu, 1997). Secondly, heptad repeats of leucines are a motif found in protein interaction

Fig. 2. Interesting motifs encoded by KARP-1. **(A)** Helical wheel diagram of the leucine repeats within the N-terminus of KARP-1. The lower-case letters correspond to the seven positions within the α -helix. The KARP-1 amino acids are shown using the single letter amino acid code starting with the first leucine at amino acid 2 (see Figure 1). **(B)** KARP-1 has homology to the Hin integrase. The amino acid sequence of both KARP-1 and Hin are aligned throughout the leucine repeat region. A consensus sequence is shown below with identical residues in upper-case and conserved residues demarcated by an asterisk (*). The following amino acids were considered similar: D, E, N and Q; F, W and Y; K and R; A and G; I, V, L, and M; S and T; C; H; P. **(C)** The hexa-heptad repeat of leucines is conserved in DNA-PK_{cs}. The amino acid sequence of both KARP-1 and DNA-PK_{cs} are aligned throughout the leucine repeat region. Identical residues are shown with a vertical line and conserved residues demarcated by an asterisk (*).

domains and over 80 such domains have been identified (Hurst, 1994). Most heptad repeats of leucines, however, are only four, or occasionally five, leucines long and a perfect hexa-heptad repeat appears, to our knowledge, to have been reported only once previously in the literature. Intriguingly, the protein with a perfect hexa-heptad repeat of leucines is DNA-PK_{cs} (Figure 2C; Hartley *et al.*, 1995), the protein with which Ku86 is known to interact (Dvir *et al.*, 1992; Gottlieb and Jackson, 1993; Suwa *et al.*, 1994). Based upon all of these observations, we thought it possible that the ORF in the 5'-UTR of Ku86 might be expressed and we therefore designated this putative novel protein, KARP-1.

The KARP-1 sequence is transcribed into poly(A)⁺ mRNA

Two experimental schemes were utilized to demonstrate that KARP-1 sequences were transcribed into mRNA. First, we prepared a radiolabeled probe corresponding to the KARP-1 sequences and performed a Northern blot analysis utilizing poly(A)⁺ mRNA isolated from human HCW2 cells (a subline of the human promyelocytic leukemia cell line, HL-60; Han *et al.*, 1996) and Sc5 cells (which will be described later) (Figure 3A). A single ~3.2 kb signal was observed in HCW2 cells' mRNA. Importantly, this pattern is different from that observed using a probe to Ku86, which results in two equally intense signals of ~2.6 and ~3.4 kb (Mimori *et al.*, 1990). The two different-sized Ku86 transcripts are apparently

the result of alternative polyadenylation as the ~3.4 kb transcript contains ~0.8 kb more 3'-UTR than the ~2.6 kb transcript (Mimori *et al.*, 1990). From this experiment we concluded that the KARP-1 region was transcribed and that the transcript was different from the normal Ku86 mRNA(s).

To confirm the above result we performed PCR with two KARP-1-specific primers and a variety of human tissue-specific cDNA libraries (Clontech, see Material and methods). As a positive control, PCR was also performed using β -actin-specific primers and all PCR products were subjected to agarose gel electrophoresis. Ethidium bromide staining of the resulting gel showed only a signal corresponding to β -actin (Figure 3B). However, when the gel was transferred onto nitrocellulose and hybridized with a KARP-1-specific probe the expected KARP-1 PCR product was observed in all human tissues except, perhaps, in skeletal muscle (Figure 3B). From this experiment we concluded that KARP-1 is probably ubiquitously expressed and that its expression level is significantly below that of the abundant β -actin message.

Mapping the 5'-end of KARP-1 by primer extension

The 5'-end of the KARP-1 message was determined by primer extension. For these experiments a radiolabeled primer complementary to the 5'-end of Ku86 was utilized (Figure 4, bottom). Two reproducible, strong bands were observed only in the presence of reverse transcriptase (Figure 4, top). These two bands were mapped to 42 bp

Fig. 3. The KARP-1 ORF is expressed. **(A)** Northern blot analysis demonstrates that the KARP-1 ORF is expressed. (Top) Poly(A)⁺ mRNA (10 µg) from HCW2 and Sc5 cells was prepared for Northern blot analysis as described in the Materials and methods. (Bottom) The probe consisted of a 328 bp fragment corresponding to the leucine (L) repeat and basic (+) region of KARP-1. **(B)** Tissue-specific library PCR demonstrates that KARP-1 is ubiquitously expressed at low levels. PCRs were performed as described in the Materials and methods using tissue-specific cDNA libraries and primers specific for either β-actin or KARP-1. The PCR products were electrophoresed onto an agarose gel and stained with ethidium bromide (EtBr). Only the 835 bp product expected from the β-actin PCR was visible (left). After the gel was transferred to nitrocellulose it was analyzed by Southern blot hybridization using a 328 bp KARP-1-specific probe. Autoradiography revealed the expected 328 bp KARP-1 PCR product (right). The name on each lane represents the human tissue from which the corresponding library was derived.

and ~400 bp in front of the Ku86 ATG. We inferred that the smaller band corresponded to the mRNA start site of Ku86 whereas the larger band corresponded to the mRNA start site for KARP-1. This interpretation was consistent with the cloning of the cDNAs for Ku86 (Mimori *et al.*, 1990) and KARP-1 (see below). In addition, from this experiment we concluded that KARP-1 and Ku86 were likely to be expressed using different promoters. This hypothesis was confirmed by mapping the genomic struc-

ture of the promoter region of the human Ku86 locus (see Figure 6) and by an analysis of the KARP-1 cDNA.

The KARP-1 cDNA

The KARP-1 cDNA was obtained using 5'- and 3'-RACE reactions and a commercially available (Clontech) human placental cDNA library constructed for PCR cloning. In each case, nested KARP-1-specific PCR primers were utilized in conjunction with nested vector primers (see

Fig. 4. Primer extension analysis reveals two predominant start sites for the Ku86 locus. Poly(A)⁺ mRNA (1 µg) was used for primer extension in either the presence (+) or absence (–) of reverse transcriptase (RT). The top panel shows a sequencing reaction which was used to size the primer extended products. The bottom panel shows a schematic of the experimental procedure.

Materials and methods). The 3'-RACE product was identical to the published human Ku86 ORF with the exception of five nucleotide changes which resulted in five amino acid changes (Figure 5). We believe that these changes most likely represent simple allelic differences. Sequence analysis of multiple 5'-RACE products revealed that two additional exons are spliced onto the leucine repeat exon, although these did not appear to be expressed as they contained in-frame stop codons and no initiator ATGs (see Figure 6). We subsequently constructed nested PCR primers corresponding to the 5'-most exon and to the 3'-UTR of Ku86. The use of these sets of primers for PCR with a cDNA library as template yielded the expected full-length cDNA, which confirmed that the aforementioned 5'- and 3'-RACE products were indeed physically connected to one another. Therefore, KARP-1 appeared

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1  MLGGNLRMLGENVRMLGRNLRRELGGNLRKLGGLRMLRVPGHGFPAPFRP
51  SRKRSGSFRYLPLVHRKRVATQVPARKKRPKRLRTGNMVRSGNKAADV
101 CVDVGFIMSNISIPGIESPFEQAKKVIITMFVQRQVFAENKDEIALVLF
151 GTDNPLSGGDQYQNTVHRHMLPDPDLLEDIESKIQPGSQADFLDALI
201 VSMVDVIOHETIGKKFEKRHIETDLSRFSKSLDIIHSLKCDISLQ
251 FFLPFSLGKEDGSGDRGDGPFRLGGHGPSFPLKGIETEQQKGLBIVKMVM
301 ISLBEGDGLDEIYSFSESLRKLKLVFKKIERHSHWPCRLTIGSNLSIRIA
351 AYKSILQERVKKIWTWVDKTLKKEDIQKETVYVCLNDDDETEVLKEDIQ
401 GFRYGSDIVPFSKVDEEQMKYKSEKCFSVLGFCKSSQVQRFFMGNOVL
451 KVFAARDDEAAVALSSLHALDDDMVAIVRYAYDKRANQVGVAFPHI
501 KHNYECLVYVQLPFMEDLRQYMFSSLKNSKKYAPTEAQLNAVDALIDSMS
551 LAKKDEKTDLTDLFPPTTKIPNPRFQRLFCQLLHRLHPRFPLPPIQOHI
601 WNMLNPPAEVTTKSQIPLSKIKTLFPLIEAKKDKQVTAQEIFQDNHEDGI
651 TAKKLKTEQGGAHFSVSSLAEGSVTSVGSVNPANFRVLVKQKKASFEEA
701 SNQLINHIEQFLDTNETPYFMKSIQCIRAFREEAIFKSEEQRFNNLLKAL
751 QEKVEIKQLNHFWEIVVDGITLITKEEASGSSVTAEAKKFLAPKDKPS
801 GDTAAVFEEGGVDLLDMI*
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Fig. 5. Predicted amino acid sequence of KARP-1. The predicted amino acid sequence (single letter amino acid code) for KARP-1 based upon 5'-RACE and 3'-RACE products is shown. The two bold methionines (M) correspond to the putative initiator methionines for KARP-1 and Ku86, respectively. The amino acids in lower-case at residues 102, 243, 650, 725 and 746 represent amino acid differences from the published human Ku86 sequence (Mimori et al., 1990).

to be composed of the leucine repeat region and the basic domain, which together comprised an additional 9 kDa of protein, appended on to the N-terminus of the Ku86 polypeptide (Figure 5).

Genomic structure of the human Ku86 locus

A compilation of the data obtained from the experiments described above allowed us to construct a map of the human Ku86 locus (Figure 6). Ku86 transcription begins 42 bp upstream of the Ku86 ATG, which resides in the exon we have designated as IIIb. There are no apparent TATA or CAAT box elements upstream of this region, but the region is very GC-rich and contains five consensus binding sites for the transcription factor Sp1 (Dyana and Tjian, 1983). The GC-rich region, which is the presumed promoter for Ku86, is, however, coincident with the KARP-1-specific ORF. The KARP-1 ORF begins at an upstream initiator ATG, located within exon IIIa. Two additional untranslated exons (I and II) are located ~1.4 and 1.2 kb, respectively, upstream from exon III. KARP-1 transcription begins just upstream of exon I. The elements which make up the KARP-1 promoter are currently under investigation.

KARP-1 protein is expressed in human cells

To confirm the above studies, we synthesized a peptide derived from the leucine repeat region of KARP-1 and used this to generate a rabbit polyclonal α-KARP-1 antibody, which was subsequently affinity purified (see Materials and methods). As a control, this antibody preparation was used to perform a Western blot analysis of extracts obtained from an *Escherichia coli* strain producing a 35 kDa fusion protein which consisted of the KARP-1 leucine repeat domain and basic region inserted into a hexa-histidine:DHFR vector. The α-KARP-1 antibody recognized a protein of 35 kDa (Figure 7A, lane 1) which appeared to be the fusion protein since this protein was not detected in extracts prepared from bacterial cells harboring just the hexa-histidine:DHFR vector (Figure 7A, lane 2). The antibody preparation was then used to perform an immunoblot analysis of whole-cell extracts prepared from human, monkey and hamster cell lines. The α-KARP-1 antibody detected a ~96 kDa protein in both primate extracts (Figure 7B, lanes 1 and 2), which was in very good agreement with the size (95 kDa) predicted

Fig. 6. The genomic structure of the human Ku86 locus. The genomic structure of the human Ku86 locus is shown in cartoon fashion. The large arrows correspond to the transcriptional start sites for KARP-1 and Ku86, respectively that were mapped by primer extension (Figure 4). The shaded and open rectangles correspond to untranslated and translated exons, respectively. The initiator methionine (ATG) for either KARP-1 or Ku86, and the leucine (L) and the basic (+) residues of KARP-1 are shown. The short arrows underscored by Sp1 correspond to binding sites for the transcription factor Sp1. The bent lines correspond to RNA splicing events deduced from cDNA cloning. The distance between exon III and exon IV and the total number of Ku86/KARP-1 exons is not known.

Fig. 7. KARP-1 protein is expressed. (A) Western blot analysis of *E. coli* strains. As a control, extracts derived from *E. coli* strains expressing either a hexa-histidine tagged KARP-1-DHFR fusion protein (lane 1) or just the hexa-histidine tagged DHFR protein (lane 2) were subjected to immunoblot analysis using the α -KARP-1-specific antibody. (B) Western blot analysis of mammalian cell lines. Whole-cell extracts were prepared from human (lanes 1, 4 and 7), monkey (lanes 2 and 5), and hamster (lanes 3 and 6) cell lines and subjected to immunoblot analysis using first the α -KARP-1-specific antibody (lanes 1–3) and subsequently the human Ku86 monoclonal antibody 111 (lanes 4–6). An additional blot was also probed with the human Ku86 polyclonal antibody 24-4 (lane 7). The locations of the 96 and 68 kDa marker proteins are shown on the far right.

from the KARP-1 cDNA (Figure 5). No signal could be detected from hamster cell extracts (Figure 7B, lane 3). As a control, the same blot was re-probed with a monoclonal antibody (mAb111) directed against human Ku86. A strong signal, which was clearly different from the size observed using the α -KARP-1 antibody, at 86 kDa was observed with human cell extracts (Figure 7B, lane 4). A very weak signal was detected with monkey extracts (Figure 7B, lane 5), and no signal was observed with hamster extracts (Figure 7B, lane 6) which suggested that the epitope recognized by antibody mAb111 is not well conserved. Disconcertingly, however, the mAb111 antibody did not cross-react with KARP-1 as was predicted from our cDNA sequence. This lack of cross-reactivity was also observed with two additional monoclonal antibodies (data not shown). Thus, to confirm these results we obtained a rabbit polyclonal Ku86 sera (Ab24-4) and utilized it in immunoblot analysis of human extracts. This antibody detected not only a protein at 86 kDa, but also one at 96 kDa (Figure 7B, lane 7), though the level of expression of the latter was several orders of magnitude lower than that of the former. From these experiments we concluded that: (i) the KARP-1 ORF was indeed translated; (ii) the protein appeared to be conserved in primates; and (iii) the level of expression of KARP-1 was significantly lower than that of the abundant Ku86 protein.

KARP-1 dominant-negative cell lines have reduced DNA-PK activity and are X-ray-sensitive

To ascertain the biological role of KARP-1, we attempted to interfere with KARP-1 function *in vivo* by overexpressing just the 88 amino acids specific to KARP-1. There was significant precedent for this approach as it was known that a number of leucine zipper proteins are negatively regulated by naturally occurring truncated

variants which retain the zipper domain, but lack other functional domains (reviewed in Foulkes and Sassone-Corsi, 1992). Thus, this region was subcloned into a mammalian expression vector and stable HCW-2 cell lines expressing this potential dominant-negative version of KARP-1 were isolated. The expression of the transfected DNA, which should result in a ~1.4 kb transcript, was confirmed by Northern hybridization in one of these subclones, Sc5 (Figure 3A).

Two subclones, Sc5 and ScD2, were tested for DNA-PK activity. Whole-cell extracts prepared from Sc5 cells showed a 2-fold reduction in DNA-PK activity compared with extracts prepared from HCW2 cells transfected with just a drug-resistance marker (Figure 8A). As DNA-PK is known to be involved in the cellular response to X-irradiation (reviewed in Jeggo *et al.*, 1995) we examined the responses of these cells to X-irradiation and observed that the Sc5 cell line was significantly more X-ray-hypersensitive than the parental HCW-2 cells (Figure 8B). Subclone ScD2 showed similar reductions in DNA-PK activity and X-ray survival as Sc5 (data not shown). To investigate whether this effect was cell type-specific, we repeated this experiment with HeLa cells. Two HeLa subclones, Sc2 and Sc8, expressing the KARP-1-specific domain also had significantly reduced DNA-PK activity and were X-ray-sensitive (Figure 8C and D and data not shown), though the X-ray hypersensitivity was not as severe as observed with Sc5 cells. HCW-2 cells are relatively radioresistant (Han *et al.*, 1995) and this may account for the X-ray hypersensitivity differences between these cell lines. From these experiments, we concluded that enforced expression of the KARP-1-specific domain resulted in a significant reduction in DNA-PK activity which manifested itself as an X-ray hypersensitivity.

Fig. 8. The phenotypes of dominant-negative subclones expressing the N-terminus of KARP-1. (A and C) DNA-PK activity is reduced in dominant-negative subclones. Whole-cell extracts were prepared from HCW-2 and Sc5 (A) or HeLa and Sc2 (C) subclones and tested for DNA-PK activity as described in Materials and methods. The results show the average of three independent experiments. (B and D) X-ray survival is reduced in dominant-negative subclones. HCW-2 and Sc5 (B) or HeLa and Sc2 (D) subclones were X-irradiated at the indicated doses and then cells surviving to form colonies were scored 2 weeks post-irradiation. The results show the average of two independent experiments, each performed in duplicate.

α -KARP-1 antibody can neutralize DNA-PK activity *in vitro*

The preceding results suggested that KARP-1 plays a role in DNA-PK activity in human cells. To confirm this, we investigated whether our α -KARP-1 antibody could neutralize DNA-PK activity *in vitro*. Whole-cell extracts were prepared from HCW-2 cells and DNA-PK assays were carried out in the presence of an irrelevant antibody (bcl-x), the α -KARP-1 antibody, an antibody to Ku86, or with no antibody addition (Figure 9). The addition of the irrelevant bcl-x antibody did not significantly affect DNA-PK activity *in vitro*. In contrast, both the α -KARP-1 and α -Ku86 antibodies significantly reduced DNA-PK activity (Figure 9) in a dose-dependent fashion (data not shown). From this experiment we concluded that neutralization of KARP-1 deleteriously affected DNA-PK activity.

Primate-specific conservation of KARP-1

The conservation of KARP-1 was assessed by Zoo blot analysis using a commercially prepared (Clontech) nitrocellulose filter and low-stringency hybridization conditions (Figure 10, left). Hybridization using a KARP-1 leucine repeat-specific probe resulted in a single strong signal at ~3.5 kb in the lane containing human DNA, though a weaker signal at ~6 kb was also observed. Interestingly, in the lane containing simian DNA, two strong signals, at ~3 kb and 4 kb, were observed. This pattern, of a single

Fig. 9. An α -KARP-1 antibody neutralizes DNA-PK activity *in vitro*. Whole-cell extracts were prepared from HCW-2 cells and divided into four equivalent aliquots. Subsequently, the aliquots were incubated for 10 min at room temperature with either no antibody, a commercial monoclonal antibody against bcl-x (Transduction Laboratories), the polyclonal α -KARP-1 antibody or the 111 monoclonal Ku86 antibody, respectively. DNA-PK assays were then carried out as described in Materials and methods. The results show the average of two independent experiments.

hybridization signal with human DNA and two signals with simian DNA was observed in several different blots using different restriction enzymes (data not shown), suggesting that the leucine repeat region has been duplicated in non-human primates. More interestingly, however, was the lack of any specific hybridization signal with any of the other genomic DNAs (the strong band observed with cow DNA corresponded to a repetitive DNA band observed using ethidium bromide staining (Figure 10, middle). To confirm that the genomic DNA in the non-primate lanes was intact, the blot was stripped and rehybridized with a probe specific for Ku86, which is known to be conserved throughout evolution (Mimori *et al.*, 1990; Porges *et al.*, 1990; Paillard and Strauss, 1991; Feldmann and Winnacker, 1993; Jacoby and Wensink, 1994; Boulton and Jackson, 1996; Errami *et al.*, 1996; He *et al.*, 1996; Lee *et al.*, 1996; Milne *et al.*, 1996). As expected, a single strong band of hybridization was observed in all lanes (Figure 10, right), except with yeast DNA, where the level of homology is probably too low to be detected by hybridization (Boulton and Jackson, 1996; Milne *et al.*, 1996). On the basis of these results we concluded that the leucine repeat region of KARP-1 may be conserved only in primates.

Sequence comparison of human and hamster Ku86 promoter regions

To extend the above observation, the promoter region of hamster Ku86 was cloned by PCR (see Materials and methods) and sequenced. A stop codon was found 27 base pairs upstream of the ATG in the hamster promoter (Figure 11), suggesting that this region is not expressed in hamsters. Interestingly, the most proximal region of the hamster promoter, which represents the basic domain of KARP-1, was—with the exception of a 17 bp insertion in the corresponding human sequence—quite well conserved between hamsters and humans (Figure 11). Most importantly, however, upstream of this region, no significant homology between the hamster and human sequences could be discerned. In particular, the leucine repeat region could not be found in the hamster sequence, confirming the Zoo blot analysis (Figure 10). From this experiment

Fig. 10. Zoo blot analysis suggests that the leucine repeat region of KARP-1 is primate-specific. A commercial zoo blot was probed under low-stringency conditions using first a probe specific for the leucine repeat of KARP-1 (left) and then Ku86 (right). The position of fragments which appear to represent specific hybridization to the probes are indicated by asterisks (*). In addition, an ethidium bromide (EtBr) staining profile provided by the manufacturer is shown (middle). The names on the top of pictures represent the species from which the genomic DNA used for the zoo blot was derived.

we concluded that KARP-1 expression is most likely restricted to primates.

Discussion

We have identified a gene, KARP-1, which appears to modulate DNA-PK activity. KARP-1 is expressed from the Ku86 locus through the use of an upstream promoter and results in the synthesis of a protein consisting of ~9 kDa of additional polypeptide appended onto the N-terminus of Ku86. KARP-1 is very unusual in that it contains interdigitating leucine repeats and appears to be expressed exclusively in primates. These observations have important implications for DNA-PK activity, mammalian DNA DSB repair and evolution.

The unusual genomic structure of the Ku86/KARP-1 locus

Ku86 is a well-characterized gene and it has long been known that the gene is ubiquitously expressed at high levels ($\sim 4 \times 10^5$ copies/cell; Mimori *et al.*, 1986). The sequence of the putative promoter region of Ku86 (Figures 1 and 6) agrees with this expression pattern. This region contains no TATA- nor CCAAT-box consensus motifs, but is GC-rich and encodes five putative binding sites for the transcription factor, Sp1 (Dyner and Tjian, 1983), consistent with the mechanism of expression of most 'housekeeping' genes (Reynolds *et al.*, 1984) and a few tissue-specific and developmentally regulated genes (Seto *et al.*, 1988). More interestingly, however, is the observation that a second, upstream promoter drives the expression of KARP-1. There is precedent for such an unusual arrangement. For example, the human gelsolin (Kwiatkowski *et al.*, 1988) and rat sterol carrier protein (Seedorf and Assmann, 1991) genes are two of a dozen known genes that produce overlapping proteins by initiating transcription from two promoters (Kozak, 1991). Thus,

the human Ku86 locus is bifunctional and belongs to a small family of loci which produce two versions of a protein.

One of the many unusual features of the KARP-1 gene is the extremely unfavorable context surrounding the putative ATG initiator (Figure 1). A compilation of vertebrate mRNAs was used to derive a consensus sequence of GCCA/GCCATGG for initiation (Kozak, 1987). While very few genes have this exact consensus, only 6/699 genes lack the purine at -3 and the G at +4 (the A of the ATG codon is designated +1, with positive and negative integers proceeding 3' and 5', respectively). Interestingly, all six of these genes encode potent growth factors, suggesting that these highly unfavorable initiation sites are a mechanism to regulate proteins which might be harmful if overproduced (Kozak, 1991). The KARP-1 initiator sequence is GTGCGCATGC and thus lacks both of these hallmark nucleotides (Figure 1). This is consistent with the low-level expression of this protein (Figure 7). We do not yet have any evidence to suggest that KARP-1 can act as a growth factor, though we are currently assessing the viability of mammalian cells overexpressing KARP-1. However, it should be noted that expression of full-length KARP-1 in bacterial cells appears toxic and consistently results in either slow-growing colonies or colonies which have inactivated or lost the KARP-1 sequence (unpublished observations).

Implications for DNA-PK activity and DNA DSB repair

KARP-1 appears to modulate DNA-PK activity. This conclusion is based upon the observation that cell lines expressing the N-terminal 9 kDa of KARP-1 had diminished DNA-PK activity (Figure 8) and that a KARP-1 polyclonal antibody could neutralize DNA-PK activity *in vitro* (Figure 9). Previously, it had been known that DNA-PK, which was believed to consist of Ku70,

	K V K K R K E I * L Q S S D T K C
Hu	.AAAGTAAAAAAGAAAGAAATCTAACTCCAGAGCTCTGACACAAAT
Ha	AGAGTAACACAGATAGAGAGTAACACAGAGTTCTTAACCACTGAGCCATCT
	R V T R * K S N Q S S * P L S H L
	L R C Y S Q * E K R D V N L * V
Hu	GCCTTAGGTGTTATTCTCAATGAGAGAAAGGACGTGAATCTTTAAGTG
Ha	CTCCAGCTCCCTGTTTAAACCAATTAATCTATTTATTAATATCCATTT
	S S S L F L T I K S I Y L I S I Y
	I K * I F K * I C R T S V S G G S
Hu	ATTAAGTGAATCTTTAAGTAAATCTGCTACTAGCGTTTCAGCGGCTC
Ha	ATTACTGTGATGTGTGGACACGTGGAGGTTAGAGGACAACCTCTTGT
	L L * C V G H V E V R G Q L L V
	N T T R P R L R R N G E N V R M
Hu	AAACACCACACGCCCCGACTACGGC...GGAATGGAGAGATGTGCGCA
Ha	GCTTCTTTGTCTCCCAAGTGAAGTCTGAAAGTCAAGGCTCTTA
	A S L S P P S E S L K S T K G P *
	L G G N L R M L G E N L R M L G
Hu	TGCTCGGCGGAATCTGCGCATGCTCGGAGAGATCTGCGCATGCTCGGC
Ha	AGACTTGATGACAAATGGCTTCACCGTAAAGCCACTTCCCTGGTCCAGT
	D L M T N G F T G K A T S L V Q Y
	R N L R E L G G N L R K
Hu	CGGA.....ATCTGCGGAGCTCGGCGGAATCTGCGCAAG
Ha	ACTAGCGTTTCTTTGTTTCTTTTAAAGCAGTCTACGAGAACAAGCCACA
	* R F V E N F K A T
	L G G N L R M L R V P G H G F P A
Hu	CTCGGCGGGAATCTGCGCATGCTCAGAGTTCGCGGCGACGGTTCCCGGC
Ha	CCTGGGAGGCTGTGCGCATGCTCAGAGCCAGGGCGGGTTCCCGGC
	P G E A V R M L T A Q G P G F P A
	P F R P S R K R S G S F R Y L P L
Hu	CCCTTTCAGGCTAGCAGGAACGAAGCGGCTCTTCCGCTATCTGCGGC
Ha	CCCTTTCAGGCTAGCAGGAGTCTAG.....GTCTGT
	P C P Q S R K S R S V
	V H R K R V A T R Q V P A R K K
Hu	TTGTCCACCGGAAGCGAGTTGCGACACGCGAGTTCCGCGCGGAAGAAG
Ha	GTGTCTCGGAACTGAGTGAACACTATAGATTCCATCC.GAAAAG
	C R P E L S * D T I D F H P K K
	R P E R L R T G N M
Hu	CGACCAAGAGCGCTGAGGACCGCAACATG
Ha	TAACCAACCGCCCTGGACGACCAACATG
	* P N R P W T S N M

Fig. 11. KARP-1 is not conserved in hamsters. The promoter region of hamster Ku86 was cloned as described in the Materials and methods. This hamster (Ha) sequence is shown in comparison with the human (Hu) sequence. Horizontal lines indicate identities and the dots represent gaps introduced into the sequences to obtain an optimal alignment. The bold sequences indicate the initiator ATGs for Ku86 and KARP-1. The predicted amino acid sequence is shown either above (human) or below (hamster) the DNA sequence and asterisks (*) indicate stop codons.

Ku86 and DNA-PK_{cs}, associated as a complex (Dvir *et al.*, 1992; Gottlieb and Jackson, 1993; Suwa *et al.*, 1994) and controlled much of the DNA DSB repair in mammals (Figure 12A; reviewed in Jackson, 1996). With the identification of KARP-1 as a component of DNA-PK, we can envision several additional models for DNA DSB repair in humans. For instance, KARP-1 may functionally replace Ku86 in the DNA-PK complex (Figure 12B) or an additional subset of DNA-PK may consist of all four components (Figure 12C). Since Ku70, Ku86 (Mimori *et al.*, 1986) and DNA-PK_{cs} (Anderson and Lees-Miller, 1992) are abundant proteins in humans and KARP-1 is apparently much rarer (Figure 7), it is likely that not all of the DNA-PK complexes are substituted by or contain KARP-1. However, it raises the possibility that the KARP-1 complexes could be utilized for special functions, such as in the repair of certain lesions, or at particular times during the cell cycle (Lee *et al.*, 1997).

We believe that, in this capacity, KARP-1 assists DNA-PK in DNA DSB repair because the dominant-negative expression of KARP-1 results in X-ray sensitivity (Figure 8B and D). Another intriguing possibility is that KARP-1 may be capable of interacting with DNA-PK_{cs} alone (Figure 12D). Since DNA-PK_{cs} appears to have no catalytic activity in the absence of DNA and Ku70 appears to predominately or exclusively contain the DNA-binding activity of the Ku heterodimer (Anderson and Lees-Miller, 1992; reviewed in Anderson and Carter, 1996) the basic region of KARP-1 may provide a DNA-binding motif for DNA-PK_{cs} (Figure 12D). In this capacity, DNA-PK_{cs} may have different targets than when it is complexed with Ku and as such may control functions other than DNA DSB repair, such as transcription or replication.

Is the KARP-1 leucine repeat region a leucine zipper?

A leucine zipper is a protein interaction domain that allows proteins to either hetero- or homodimerize (Landschulz *et al.*, 1988), or occasionally tetramerize (Harbury *et al.*, 1993). The two repeated leucine motifs found in KARP-1 independently conform to the heptad reiterations of leucines found in leucine zippers (reviewed in Hurst, 1994). Interdigitating motifs of leucines—such as in KARP-1—have, to our knowledge, not been described before in naturally occurring proteins. Intriguingly, however, such motifs have been constructed *in vitro* and these proteins tend to trimerize (Harbury *et al.*, 1993). Thus, KARP-1, alone or in connection with DNA-PK_{cs}, may form a much larger complex mediated by leucine zipper protein-protein interactions.

Alternatively, the KARP-1 leucine repeats may not form a leucine zipper(s). In particular, glycine and proline residues are generally not found in leucine zippers because the residues are either too small or too large, respectively, to maintain the amphipathic α -helix. The KARP-1 sequence, however, is rich in glycine residues (Figure 2A). In addition, many leucine zipper proteins contain a second motif, the basic region, which is thought to provide sequence-specific DNA binding, and which is invariably found on the N-terminal side of the zipper motif (the 'bZIP' motif; Landschulz *et al.*, 1988). While some leucine-repeat proteins (e.g. DNA-PK_{cs}; Hartley *et al.*, 1995) completely lack the basic region (reviewed in Hurst, 1994), KARP-1 would be the first protein, to our knowledge, which contained a basic region on the C-terminal side of the zipper motif (a 'ZIPb' protein). Lastly, and perhaps most importantly, KARP-1 showed homology to the Hin integrase, a protein which homodimerizes and participates in bacterial DNA recombination (reviewed in Plasterk and Van De Putte, 1984). In particular, many of the leucine residues are conserved between the two proteins (Figure 2B). While the structure of Hin has not yet been determined, the structure of a very related protein, $\gamma\delta$ resolvase, showed that the leucine repeat region formed a hydrophobic core rather than an α -helix, and that it was not involved in the dimerization of $\gamma\delta$ resolvase monomers (Yang and Steitz, 1995). Thus, while the KARP-1 leucine repeat region may form a leucine zipper, confirmation of this will require experimental evidence.

Fig. 12. Hypothetical models for KARP-1 regulation of DNA-PK. (A) A cartoon showing the previously accepted model of DNA-PK. (B) KARP-1 may replace Ku86 in some of the DNA-PK complexes. (C) KARP-1 may associate with the other three components of DNA-PK and alter or augment the activity of DNA-PK. (D) KARP-1 may provide a new DNA binding subunit for DNA-PK_{cs} which may result in novel activities such as transcription, DNA replication or chromatin reorganization.

Primate-specific expression of KARP-1

An additional intriguing feature of KARP-1 was that the gene appeared only to exist and be expressed in primate cells, as demonstrated by Zoo blot (Figure 10) and DNA sequencing (Figure 11) analyses. Primate-specific repetitive DNA families such as Alu (Jelinek and Schmid, 1982) are well known. Primate-specific DNA capable of encoding functional proteins, however, is much more unusual, though not unique. For example, the protein which binds to the 7SL RNA component of the signal recognition protein is 4 kDa larger in humans than in rodents due to a trinucleotide expansion (which encodes a polyalanine stretch) in the gene (Chang *et al.*, 1994). In addition, a putative primate-specific insertion near the hinge region of the T-cell receptor α chain gene has also been reported (Thiel *et al.*, 1995). These reports notwithstanding however, the existence of KARP-1, which may have biochemical properties distinct from the well-conserved Ku86 protein, would seem to have important evolutionary repercussions.

First, where did the primate-specific leucine repeat region originate? This sequence consists of seven amino acids (MLGGNLR) imperfectly repeated five times. One possibility is that the leucine repeat region arose by expansion of this minimal seven amino acid domain, much in the way the primate 7SL binding protein has expanded through trinucleotide expansion, and subsequently has acquired a beneficial function. Intriguingly, we have recently identified a human cell line which contains this

motif repeated six times, thus generating a leucine repeat region containing interdigitating hepta-heptad and hexa-heptad repeats of leucines (unpublished data). Thus, the leucine repeat region appears to be polymorphic and potentially unstable in humans.

Secondly, why do primates need two versions of Ku86? As noted above, KARP-1 may contain growth factor activity which may be uniquely needed by primates. Alternatively, the only demonstrated role for KARP-1 is in modulating DNA-PK activity (Figures 8 and 9). Interestingly, it has been shown independently that primate cells contain 50-fold more DNA-PK activity than rodent cells (Finnie *et al.*, 1995). While this difference has been interpreted as a difference in the level of expression of the DNA-PK subunits between primates and rodents (Finnie *et al.*, 1996), the existence of primate-specific KARP-1 provides an additional explanation. This begs a second question, however, of why primates should require elevated levels of DNA-PK, especially as rodent cells appear to tolerate radiation damage and repair DNA DSBs as well as primate cells. One of the obvious differences between primates and rodents is that primates generally have a much longer lifespan. The obvious benefits of longevity, however, may be offset by the extended opportunity to incur DNA damage leading to toxic or mutagenic events. Thus, long-lived primates may need elevated levels of DNA repair, whereas the relatively short-lived rodents can survive with reduced levels. It will be interesting to test whether the expression of human KARP-1 in rodent cells results in increased DNA-PK activity.

Lastly, it should be pointed out that the biochemical function of KARP-1 may be conserved in non-primate mammals. Though the leucine repeat region of KARP-1 appears to be primate-specific (Figures 10 and 11) a KARP-1-like activity in non-primate mammals may be expressed, through an as of yet unidentified mechanism, from the Ku86 locus. Intriguingly, mouse strains in which Ku86 had been inactivated by deletion of internal exons showed an unexpected growth defect in addition to the expected immune deficiency and X-ray hypersensitivity (Nussenzweig *et al.*, 1996). Thus, adult Ku86-knockout mice are only half the size of their wild-type littermates (Nussenzweig *et al.*, 1996). Consequently, the deletion of internal exons of the murine Ku86 locus may have inactivated two genes: Ku86, which resulted in the immune deficiency and, inadvertently, a KARP-1-like gene, which resulted in the growth defect. These results suggest that the genomic locus and gene expression patterns of murine Ku86 should be investigated in greater detail.

Materials and methods

Isolation of KARP-1 genomic clones

The genomic DNA 5'-upstream of the Ku86 initiator ATG was obtained by PCR using a GenomeWalker Kit (Clontech). Primary PCR utilized the Ming5 (5'-CTCATGGTAAAGCCACGTCCATACACAGC-3') and AP-1 primers (5'-GTAATACGACTCACTATAGGGC-3') and secondary PCR utilized Ming4 (5'-AACAGCTGCCTATTCCCCGACCGCACAT-3') and AP-2 (5'-ACTATAGGGCAGCGTGGT-3') primers. The ~1.2 kb resulting PCR fragment was subcloned using a TA cloning vector (Invitrogen) and sequenced. This information was utilized to generate additional PCR primers, Ming14 (5'-TCTTGTCAGTGGTCCTTGCTCCCTTCTTG-3') and Ming15 (5'-TACGATCCAGAGCTAGGAGAGAGAGAAAA-3'), homologous to 5'-distal sequences and the process was repeated until ~4.0 kb of genomic DNA was subcloned. Sequence analyses were performed either with DNASIS (Hitachi) or the GCG computer program (University of Wisconsin).

Isolation of hamster Ku86 genomic clones

To clone the promoter region of the hamster Ku86 gene, genomic DNA from the hamster lung V79-4 cell line (Lee *et al.*, 1995) was digested with *Xba*I and ligated with pBluescript DNA which had been digested with *Xba*I and *Xho*I. This ligation mixture was then used for PCR. Primary PCR with carried out with SEL86-2 (5'-AGCTGCCTATTAGCGGACCACGCCATTGTT-3') and a commercially available T7 primer while secondary PCR was performed with SEL86-1 (5'-GCTGGTCCACGGGCGGTTTGGTTACTTTT-3') and the same T7 primer. All PCR products were subcloned into a TA cloning vector (Invitrogen). DNA sequencing was carried out using an automated sequencer and data was obtained for both strands.

Isolation of KARP-1 cDNA clones

Partial KARP-1 cDNA clones were obtained using 3'- and 5'-RACE reactions. All RACE reactions utilized a Marathon cDNA Amplification Kit (Clontech) and vector primers AP-1 (5'-CCATCCTAATACGACTCACTATAGGGC-3') and AP-2 (5'-ACTCACTATAGGGCTCGAGCGGC-3'). These primers were utilized in conjunction with four gene-specific primers. 3'-RACE reactions utilized KJ1011 (5'-ACGGCGGAATGGAGAGAATGTGCGCATGC-3') and KJ15 (5'-CCTTTCA-GGCCTAGCAGGAAACGAAGCGGC-3') while 5'-RACE reactions utilized KJ13 (5'-CGTTTCTGTCTAGGCCTGAAAGGGGC-3') and LZ5E (5'-GCCCGAGCATGCGACATTCTCTCCA-3') primers. A full-length cDNA was obtained from the same library using KJ007 (5'-GCTGGACCTGGTGGCACACCTGTGGTCC-3') and KJ008 (5'-GAATCCAGCATCAGCGATGGCAGCTC-3') for primary PCR and KJ003 (5'-GGGAGACAAGGACCACTGACAAGATA-3') and 86-7 (5'-ATACAGCTGCTGTGTCTCCACTTGG-3') for secondary PCR. PCR products were subcloned into a TA cloning vector (Invitrogen). DNA sequencing was carried out using an automated sequencer and data was obtained for both strands.

Northern blot analysis

Poly(A)⁺ RNA was isolated from HCW2 and Sc5 cells using a FastTrack mRNA isolation kit (Invitrogen) and 10 µg was electrophoresed on a 1.2% agarose gel. The gel was subsequently incubated in 0.5 M NaOH for 15 min and neutralized in 20× SSC for 40 min. mRNA was transferred onto nitrocellulose by capillary transfer and hybridized in 50% formamide, 5× Denhardt's solution, 5× SSPE, 0.1% sodium dodecyl sulfate (SDS), 100 µg/ml herring sperm DNA, and 2% polyethylene glycol (PEG) 8000 with a 328 bp KARP-1-specific probe generated by PCR using the primers KJ10 (5'-GCTCAAACACCACAC-GCCCC-3') and Ming1 (5'-TTATTCCCCGACCGACCAT-3') and radiolabeled by random priming (Stratagene).

Tissue-specific library PCR

Library PCR was carried out using human tissue-specific cDNA libraries (Clontech) as templates. KJ10 and Ming1 primers were used for the PCR reactions. As a control, β-actin PCR primers, provided by the supplier, were also used. PCR products were separated by electrophoresis on a 1.0% agarose gel and transferred onto nitrocellulose. Southern hybridization of this filter was performed in 50% formamide, 5× Denhardt's solution, 5× SSPE, 0.1% SDS, and 100 µg/ml herring sperm DNA with the 328 bp KARP-1-specific probe described above.

Primer extension

Primer extension was performed with 1 µg of poly(A)⁺ RNA. RNA and an end-labeled primer (Ming4) were incubated at 65°C for 2 min and slowly cooled to 37°C. Then, 100 units of MuLV reverse transcriptase, buffer (50 mM Tris-HCl, pH 8.3, 8 mM MgCl₂, 10 mM dithiothreitol), and 50 nmol dNTP mix were added and reaction mixtures were incubated for 1 h at 37°C. The reaction products were then separated by electrophoresis on a 6% acrylamide gel with 8 M urea and subjected to autoradiography.

Antibody generation and Western blot analysis

A rabbit KARP-1 polyclonal antibody was generated using a KARP-1-specific peptide (N'-GRNLRGLGGLRKLGG-C') conjugated with KLH (Keyhole Limpet hemocyanin) protein (Bio-Synthesis, Inc.). After three boosts, serum was obtained from the rabbit and α-KARP-1-specific antibody was affinity-purified by elution through a CNBr-Sepharose column (Pharmacia) conjugated with the same KARP-1-specific peptide. α-KARP-1 antibody was eluted with glycine-HCl, pH 4.5, neutralized by adding one-hundredth volume of 1 M Tris-HCl, pH 7.0, and then used for further study. For Western blot analyses, the purified antibody was diluted 1 to 100-fold. Western blotting was performed with the ECL system (Amersham) with 20 µg of whole-cell extract in each lane.

Generation of a KARP-1 fusion protein

A KARP-1 fusion protein was generated by using the hexa-histidine: DHFR (dihydrofolate reductase) tag expression system (Qiagen). An ~300 bp PCR fragment containing the KARP-1-specific region was generated using Ming1 and FP (5'-AGATCTAGAGAATGTGCGCATGC-3') primers and then subcloned into the TA vector. The fragment was subsequently removed by digestion with *Bgl*II and *Pvu*II and recloned into the pQE41 vector. The induction of the fusion protein was performed according to the manufacturer's protocol.

Cell culture

The HCW-2 cell line was derived from the human promyelocytic leukemia cell line, HL60 (Han *et al.*, 1996). HCW2 cells were cultured in RPMI media (Gibco) with 10% fetal calf serum (Gibco). HeLa (human) and CV-1 (monkey) and cells were grown in DMEM media (Gibco) with 10% fetal calf serum. V79-4 (hamster) cells were grown in α-MEM media (Gibco) with 10% fetal calf serum. Transfection of HCW-2 and HeLa cell lines was achieved by electroporation and included the eukaryotic expression vector SRα containing the N-terminus of KARP-1 and a drug resistance marker, hygromycin (He *et al.*, 1996). Independent hygromycin-resistant colonies were either isolated by limiting dilution (HCW-2) or by toothpicking (HeLa). Individual colonies were expanded and subsequently tested for DNA-PK activity and X-ray survival.

X-ray survival

Cell lines were irradiated with a cesium source (11 Gy/min) at a variety of doses. After irradiation, cells were either replated in 96-well plates (HCW-2 cells and subclones) or onto 100 mm tissue culture plates (HeLa cells and subclones) at defined cell densities. Cells were cultured

for 2 weeks and cells surviving to form colonies were counted (Hendrickson *et al.*, 1991).

DNA-PK assays

DNA-PK assays were performed as described (Anderson and Lees-Miller, 1992; Lee *et al.*, 1997). Twenty μ g of whole-cell extract was used per DNA-PK assay. The specific activity of DNA-PK was calculated as the DNA-PK activity generated with DNA minus the DNA-PK activity generated without DNA.

Zoo blotting

A nitrocellulose filter for Zoo blot analysis was purchased from Clontech. Low-stringency hybridization was performed in 20% formamide, 5 $\times$ Denhardt's solution, 5 $\times$ SSPE, 0.1% SDS, and 100 μ g/ml herring sperm DNA at 42°C with a 230 bp *SacI* restriction fragment which encompasses the leucine repeat region of KARP-1. The blot was subsequently stripped by incubating in 0.1 $\times$ SSC and 0.1% SDS at 94°C for 30 min. The blot was subsequently reprobed with a 323 bp *PstI*-*ClaI* fragment derived from a human Ku86 cDNA.

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