Characterization of the RNA Binding Properties of Ku Protein†

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ABSTRACT: Ku protein, a heterodimer of 70 and 83 kDa polypeptides, is the regulatory component of the DNA-dependent protein kinase (DNA-PK). Ku protein binds to DNA ends and is essential for DNA double-strand break repair and V(D)J recombination. Although there is some evidence that Ku protein also binds RNA, its RNA binding properties have not been systematically explored. In the present study, Ku-binding RNAs were identified using systematic evolution of ligands by exponential enrichment (SELEX) technology. These RNAs were assigned to three classes based on common sequence motifs. Most of the selected RNAs bound to Ku protein with a $K_d \leq 2$ nM, comparable to the affinity of DNA fragments for Ku protein under similar conditions. Many of the RNAs inhibited DNA-PK activity by competing with DNA for a common binding site in Ku protein. None of several RNAs that were tested activated DNA-PK in the absence of DNA. The identification of diverse RNAs that bind avidly to Ku protein is consistent with the idea that natural RNAs may serve as modulators of DNA-PK activity. Moreover, the RNAs identified in this study may have utility as tools for experimental manipulation of DNA double-strand break repair activity in cells and cell extracts.

Ku protein, a heterodimer of 70 and 83 kDa polypeptides, was first identified as an autoantigen in sera from certain patients with autoimmune disease (*1*). Subsequent characterization showed that Ku protein binds avidly to double-stranded DNA ends and other structural discontinuities in DNA such as nicks, gaps, and hairpins (*2*−*6*). Further biochemical analysis demonstrated that Ku protein is the regulatory component of the DNA-dependent protein kinase (*7*, *8*). In the presence of DNA ends, Ku protein can interact with the catalytic subunit of DNA-PK,[1] which is thereby targeted to the DNA. The ability of Ku protein to interact with DNA ends suggested that Ku and DNA-PKcs may play a role in DNA repair and recombination (*9*). Subsequent characterization of ionizing radiation-sensitive mutant cell lines showed that Ku protein and DNA-PKcs are essential for repair of DNA double-strand breaks and for V(D)J recombination (*10*−*18*).

The binding of Ku protein to double-stranded DNA ends is largely sequence-independent. The ability of Ku protein to undergo facilitated transfer between DNA fragments with cohesive ends suggests that Ku protein may be able to interact transiently with two DNAs simultaneously, perhaps serving to align the ends for ligation (*19*). Consistent with this, recent atomic force microscopy and electron microscopy studies show images of Ku protein tethering DNA fragments together and participating in loop structures (*20*, *21*). There have also been a number of reports of possible sequence-specific binding of Ku protein to DNA (for example, refs *22*−*25*). Most recently, a sequence in the long terminal repeat of mouse mammary tumor virus has been characterized that appears to allow interaction of Ku protein with DNA in the absence of ends or single-stranded regions (*26*, *27*).

There is some evidence that Ku protein interacts with RNA, although this has been much less studied than the interaction with DNA. Antibodies to Ku protein stain both the nucleoplasm and the nucleolus. The amount of Ku protein in the nucleolus changes depending on the growth state of the cell, suggesting that this localization is actively regulated (*28*). Separately, it has been demonstrated that nucleolar staining is sensitive to RNase treatment, whereas nucleoplasmic staining is not (*29*). Thus, nucleolar localization may be regulated by interaction of Ku protein with RNA. Ku protein does not appear to bind to bulk tRNA or to synthetic RNA polymers (*2*). However, one study showed that Ku protein forms a specific complex with an RNA that included the HIV trans-activation response (TAR) element sequence (*30*).

In the present study, we have systematically investigated the RNA binding properties of Ku protein using SELEX (*s*ystematic *e*volution of *l*igands by *ex*ponential enrichment) technology (*31*). With this technology, it is possible to identify RNAs from a large, random pool that bind to a ligand of interest. The SELEX method has been used to identify RNAs that bind to nucleic acid binding proteins, to nonnucleic acid binding proteins, and to small molecules (reviewed in ref *32*). We have used the SELEX procedure to isolate 19 different aptamer RNAs that bind avidly to Ku protein. Many of the RNAs contain common sequence motifs. A number of the RNAs bind competitively with

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[1] Abbreviations: DNA-PK, DNA-dependent protein kinase; cs, catalytic subunit; PCIA, phenol−chloroform−isoamyl alcohol (25:24:1 v/v/v); DTT, dithiothreitol; TE, 10 mM Tris-HCl, pH 7.9, and 1 mM EDTA; TCA, trichloroacetic acid; HIV, human immunodeficiency virus; SELEX, systematic evolution of ligands by exponential enrichment.

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Table 1: Summary of the Selection Parameters

	selection I				selection II			
SELEX round	input RNA (nM)	Ku protein[a] (nM)	KCl[b] (mM)	selection[c] method	input RNA (nM)	Ku protein (nM)	KCl (mM)	selection method
1	1305	64.1	50	NCFA	1330	53	120	EMSA
2	612.5	24.3	50	EMSA	1660	42	120	EMSA
3	860	42.6	120	EMSA	456	14.9	150	EMSA
4	1130	42	120	EMSA	144	3.84	160	EMSA
5	2320	64	150	EMSA	1065	21.3	160	EMSA
6	142	3.2	160	EMSA	2710	18.6	160	EMSA
7					436	2.4	160	EMSA

[a] The ratio of Ku protein to input RNA was gradually decreased in order to increase the stringency. [b] Salt concentration was increased to maintain high stringency. [c] Nitrocellulose filter binding assay (NCFA) or eletrophoretic mobility shift assay (EMSA) were employed for selection methods.

DNA to Ku protein and thereby inhibit DNA-PK enzymatic activity.

EXPERIMENTAL PROCEDURES

Protein Purification. Native DNA-PKcs and Ku protein were purified from HeLa cell nuclear extracts as previously described, except that the phenyl-Superose and Mono S steps were omitted (*33*). Monoclonal antibodies to Ku protein were obtained from Neomarkers (Fremont, CA).

Selection of Ku-Binding RNAs. SELEX procedures were carried out largely as described (*34*). The template for synthesis of the starting RNA pool was based on a DNA oligonucleotide, 5′-CCCGGATCCTAGTTCACGATGCT-GCAA-$(N)_{40}$-TTACGGTCTGAGAAAATATCCTCCC-3′, where N indicates an equimolar mixture of A, G, C, and T. DNA templates were generated by PCR amplification of this oligonucleotide using primer 1, 5′-CCCAAGCTTAATAC-GACTCACTATAGGGAGGATATTTTCTCAGACCGTAA-3′, and primer 2, 5′-CCCGGATCCTAGTTCACGATGCT-GCAA-3′. Amplification was carried out with 0.5 μM oligonucleotide containing the random segment, 2 μM primer 1 and primer 2, 1 mM dATP, dCTP, dGTP, and dTTP, 50 units/mL Taq DNA polymerase, 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 7.5 mM $MgCl_2$, and 50 μg/mL BSA in a volume of 0.5 mL. After four cycles of amplification (93 °C for 30 s, 57 °C for 20 s, and 73 °C for 90 s), the reaction was extracted with an equal volume of PCIA, and nucleic acids were precipitated from the supernatant by addition of 0.1 volume of 3 M NaOAc, pH 5.2, and 2.5 volumes of EtOH. This template preparation was incubated for 2−3 h at 37 °C in a reaction mixture containing 475 units/mL T7 RNA polymerase, 40 mM Tris-HCl, pH 8.0, 12 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 0.002% Triton X-100, 4% PEG 8000, 2 mM ATP, UTP, and GTP, and 0.2 mM [α-^{32}P]-CTP (1 Ci/mmol) in a final volume of 200 μL. DNase I was added (25 units/mL) and incubation was continued for 30 min. The resulting RNAs were fractionated by 10% urea−PAGE, and gel slices containing full-length 88 nt RNA were excised, crushed, mixed with 0.5 mL of TE, 0.5 mL of phenol, and 10 μL of 10% sodium dodecyl sulfate, and tumbled overnight at 4 °C to extract RNA. The supernatant was collected and RNA was precipitated with NaOAc and EtOH as described above. RNA was dissolved in TE, denatured at 100 °C for 2 min, and renatured by adjusting to 5 mM $MgCl_2$, cooling rapidly to 0 °C, and incubating for 30 min. The amount of RNA recovered was estimated by liquid scintillation counting.

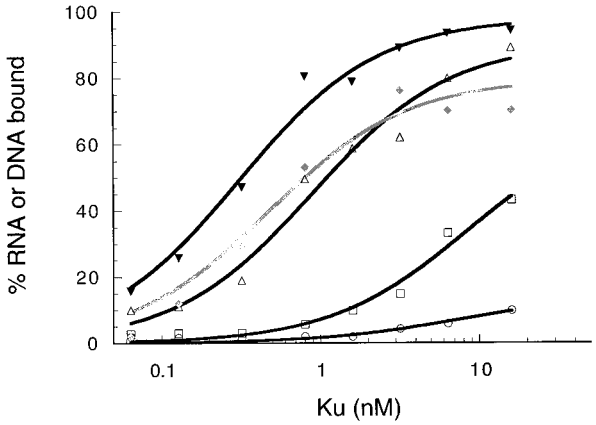

FIGURE 1: Representative Ku protein−RNA binding curves after successive rounds of SELEX. RNA and DNA probes were prepared as described in Experimental Procedures. Binding curves were obtained with an electrophoretic mobility shift assay (EMSA), followed by a nonlinear least-squares fit of the data. Radioloabeled nucleic acid probes are as follows: (□) unselected RNA; (△) RNA after fourth round of selection; (▼) RNA after sixth round of selection; (○) HIV-TAR RNA (included for comparison); (◆) 21 bp double-stranded DNA.

To perform in vitro selections, RNA and Ku protein were incubated for 30 min at room temperature in a buffer containing 25 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, 0.5 mM DTT, and 0.01% Tween 20 in a final volume of 50−100 μL. Concentrations of RNA, Ku protein, and KCl were variable, as specified in Table 1 and in the legend to Figure 1. Ku protein−RNA complexes were isolated by electrophoresis on a 5% nondenaturing polyacrylamide gel containing 25 mM Tris-HCl, pH 8.3, 190 mM glycine, and 1 mM EDTA. In one experiment, complexes were isolated by passage over a nitrocellulose filter (Millipore 25-μm HAWP), which was washed twice with 5 mL of 25 mM Tris-HCl, pH 7.9. RNA was eluted from the polyacrylamide gel or the filter by tumbling in a mixture of TE, phenol, and SDS as described previously.

When additional rounds of selection were performed, the selected RNA was reverse-transcribed by incubating in a reaction mixture containing 50 mM Tris-HCl, pH 8.3, 60 mM NaCl, 6 mM $Mg(OAc)_2$, 10 mM DTT, 0.4 mM dATP, dCTP, dGTP, and dTTP, and 300 units/mL AMV reverse transcriptase, in a final volume of 50 μL. The resulting cDNA was amplified by 15 cycles of PCR using primers 1 and 2, and this template was used to generate a new pool of RNA transcripts as described previously. Additional rounds

of selection and amplification were performed as described in Table 1.

After six or seven rounds of selection, cDNA was synthesized from the final RNA pool and amplified by five cycles of PCR using primers 1 and 2. The product was digested with *Bam*HI and *Hin*dIII and subcloned into the *Bam*HI and *Hin*dIII sites of pGEM3zf(+) (Promega) or pZero2.1 (Invitrogen). Inserts were sequenced with an M13 reverse primer using an ABI automated sequencer.

Pools of RNA at various stages of selection were also characterized directly for their ability to bind to Ku protein in an electrophoretic mobility shift assay. For comparison, these assays also included a HIV TAR RNA, which was synthesized by T7 RNA polymerase as described in the preceding section. This RNA consisted of the following HIV-derived sequence: GGGUCUCUCUGGUUAGACCA-GAUCUGAGCCUGGGAGCUCUCUGGCUAAC-UAGGGAACCC. Comparison was also made with a 21 bp double-stranded DNA: CTCAGGCGTTGACGACAACCC and its complement.

Characterization of Aptamer RNAs. Individual aptamer RNAs were synthesized by T7 RNA polymerase using linearized plasmid template. Radiolabeled RNA was prepared using the conditions described in the preceding section, and nonradiolabeled RNA was prepared using a MEGAshortscript T7 kit (Ambion) with 8 μg of linearized plasmid template. The RNA was gel-purified, heated, and refolded as described in the preceding section, and the final concentration was determined spectrophotometrically. Secondary structures were predicted using the method of Zuker (*35*, *36*) as implemented on the mfold server (www.ibc.wustl.edu/%7Ezuker/rna/form1.cgi).

Kinase Assay. Peptide phosphorylation was carried out using a previously described method (*37*) with modifications. Reactions contained 1 nM Ku protein, 0.5 nM DNA-PKcs, 100 μM p53-derived peptide (EPPLSQEAFADLLWKK, phosphorylation site underlined), 0.25 nM DNA fragment [308 bp *Bgl*I−*Blp*I fragment from pHSE$_1$ (*38*)], 4000 units/mL RNasin (Promega), 0.2 mM [γ-^{32}P]ATP (1.25 Ci/mmol), 25 mM Tris-HCl, pH 7.9, 25 mM $MgCl_2$, 1.5 mM DTT, 50 mM KCl, and 10% glycerol in a total volume of 20 μL. Reactions were incubated at 30 °C for 45 min, stopped by addition of 10 μL of 1 mg/mL BSA and 10 μL of 40% TCA, and further incubated for 30 min on ice. Precipitated protein was removed by centrifugation and 10 μL of each supernatant, which contained the phosphorylated p53 peptide, was spotted on a phosphocellulose filter (Whatman P81). The filters were washed three times with 15% HOAc for 15 min, and incorporation of radiolabel was measured by liquid scintillation counting.

RESULTS

Isolation of RNAs That Bind to Ku Protein. Because there was little previous information about Ku protein−RNA interactions, we began our studies by screening for Ku-binding RNAs in a large, unbiased pool, which contained about 10^{14} different RNA sequences. The RNAs contained a 40 nt random region in the center, flanked by constant regions at each end. Purified Ku protein was mixed with the RNA pool and allowed to bind, as described in Experimental Procedures. Ku−RNA complexes were isolated using either a nitrocellulose filter binding or an electrophoretic mobility shift assay. RNA that was bound to Ku protein was isolated, amplified by reverse transcriptase−polymerase chain reaction (RT-PCR), and again selected for its ability to bind to Ku protein. This process was repeated 6−7 times. Selection conditions used for each round of selection are given in Table 1. To maintain the stringency of selection, the RNA:protein ratio, the KCl concentration, or both were progressively increased. Two independent selections were conducted using different batches of starting RNA.

To follow the progress of this SELEX procedure, RNA was prepared from the unselected pool and after the fourth and sixth rounds of selection. Binding to Ku protein was measured using an electrophoretic mobility shift binding assay, as shown in Figure 1. Ku protein bound to unselected RNA with an apparent average K_d of 24 nM. This binding was somewhat stronger than expected, given that previous work had found that Ku protein had little ability to interact with tRNA or synthetic RNA polymers (*2*). Pooled RNA tested after the fourth and sixth rounds of SELEX showed an increased ability to bind to Ku protein. RNA obtained after the sixth round of selection bound with an apparent K_d of about 0.3 nM, which was comparable to the binding seen with a double-stranded DNA oligonucleotide under similar conditions. Although the average affinity of the selected RNA was only 75-fold greater than for the unselected pool, subsequent experiments revealed clear functional differences, as only the selected RNAs were able to inhibit biochemical activities of Ku protein.

For comparison, we also prepared RNA containing the TAR sequence from HIV-1. TAR-containing RNAs have previously been reported to bind selectively to Ku protein (*30*). Binding of the TAR RNA was measurable, but weak, under the conditions of our experiments. These results confirm that TAR-containing RNA has some ability to bind to Ku protein but indicate that the binding is not particularly strong, relative to other short RNAs. An in vitro yeast tRNA transcript also bound very weakly to Ku protein (data not shown).

Cloning, Sequence Analysis, and K_d of Aptamers. After six and seven rounds of SELEX (selections I and II in Table 1, respectively), the pooled RNA was reverse-transcribed, PCR-amplified, and cloned into plasmid vectors as described in Experimental Procedures. A total of 82 clones was isolated, including 63 from selection I and 19 from selection II. Allowing for duplicates, the 82 clones represented 19 independent sequences, which are given in Table 2. An equilibrium dissociation constant was determined for the binding of each of the 19 different RNAs to Ku protein. The results, given in Table 2, showed that most of the RNAs have a K_d of less than 2 nM.

Sequence alignment revealed that the RNAs can be grouped into three classes, based on the presence of conserved sequence motifs. The most striking of these motifs was a sequence, GCUUUCCCANNNAC, that was perfectly conserved in three independent RNA sequences and partially conserved in four others. A second motif, A(A/C)AUGA, was perfectly conserved in three RNAs and partially conserved in seven others. A third motif, which partially overlaps the second, had the sequence AACUUCGA. This motif was present in one RNA and partially conserved in

Table 2: RNA Sequences and K_d Values from the SELEX Procedure

name (frequency)	aligned sequence[a]	K_d	DNA-PK[b] inhibition
CLASS I			
SC6 (3)	GACUCACGAUGGACCAUACGCCUUCCCACUGGUCUUGUUA	2.0 nM	C
#1-2 (1)	CAACACCUUGCUUUCCCAAUACCCUGAAAUACAGUCGGAU	1.5 nM	A
#1-17 (1)	UCCUUAUUUUAUGGCUUUCCCACGCACACAAGCGUCUGCG	3.2 nM	B
#85 (1)	CAAGUAUCACGCACUUUCCCAUUCACUGUUAGAGACUGA	0.7 nM	B
HYBRID			
#2 (1)	GCCUAUGCACGGAGCUUUCCCAGCUACAGAUGAAACCAGC	0.3 nM	A
SC4 (8)	CCUAGUCUAAUCGAGGCUUUCCCAGUGAC-AAUGACCCAC	1.7 nM	A
SC5 (4)	CUUGAACAUGAUAG-GCUUACCCAUAGACAGAUUGACCCUU	2.0 nM	A
CLASS II			
SC9 (3)	UGCCUUUAGCUGCGAC-AAUGAACAGCAUGACCUCACUAC	0.6 nM	B
SC8 (8)	GUCCUUCACUAAUGCUUACCAGACACACUAAGAACGUCAC	0.6 nM	A
SC3 (3)	CAUUACCACAGUUCUAGCAUCCCGCAAUGGUAAGUCCGCA	0.8 nM	B
SC1 (23)	UUGUUCAACCUUGUCUAACAUGAUACCGAUACGGACUACA	1.2 nM	B
#84 (1)	AUCCGCGUACCGGGCUCAAAUGUCACUAUAGUAGAAAGCA	1.8 nM	B
#52 (1)	CUGAUCGUUCAAUGACUAUUCUUUACCUUGAGUAACCGA	3.2 nM	C
HYBRID			
SC12 (4)	CUCGCAACAUGACUUCGAAAGUUUAAUCGUUCUUGUCAA	0.5 nM	B
CLASS III			
#7-3 (1)	AGGUCGGCAUACAGAGUUCCGAAUGCGCGUAACUUCGACU	1.8 nM	A
SC11 (2)	CUUAGUUUCGAUCGAAGCUCAUUGGCCCAGCGUGGAUAAC	>10 nM	B
SC2 (12)	CACGCUCUACAACAGAUUGCGAAUUAACUUACGCUUCAUA	0.8 nM	A
#42 (1)	CAUCCUGGUACUCACUUCGACAUCGUACGUUCAAUCAUAC	4.5 nM	B
OTHER			
SC13 (3)	ACCUUUUUAGACGAACCUCAAAGUACAUUUAGUUGAAAAC	0.8 nM	B

[a] RNA sequences are aligned to show maximum sequence identity. The underlined sequences indicate sequence identity within the class. [b] DNA-PK inhibition is classified according to the ability of the aptamer to inhibit DNA-PK enzymatic activity when present at ≥16 nM in standard reaction. A, more than 85% inhibition; B, 30−80% inhibition; C, less than 30% inhibition.

four others. Several RNAs fell into hybrid classes containing two of the three motifs. Subsequent analysis showed that the hybrid RNAs containing two motifs were among the most efficacious blockers of Ku protein activity in functional assays.

Inhibition of DNA-PK Activity by Different RNA Aptamers. We were interested in determining whether the RNAs that bound to Ku protein were capable of regulating Ku protein activity. One of the major biochemical functions of Ku protein is to regulate the activity of DNA-PK. Ku protein targets the catalytic subunit of DNA-PK to DNA, increasing phosphorylation activity by 5−50-fold (*7*, *8*). Under some assay conditions, DNA-PK that has been depleted of Ku protein can no longer be stimulated by double-stranded DNA (*33*).

We examined whether the Ku-binding RNAs were able to block the ability of Ku protein to activate DNA-PK. The 19 selected RNAs, as well as RNA from the unselected pool, were prepared by in vitro transcription and were added to a DNA-PK phosphorylation assay. The effect on DNA-PK activity was measured relative to control reactions with no added RNA. Unselected RNA had no effect on DNA-PK activity (Figure 2A). Of the selected RNAs, seven inhibited DNA-PK activity by 85% or more when present at 16−32 nM (Figure 2A). Ten RNAs inhibited DNA-PK to a lesser extent and two had no effect on DNA-PK in the concentration range tested (data not shown; see Table 2). These results indicate that at least some of the selected RNAs interact with regions of Ku protein that are critical for its biochemical activity. Since DNA-PKcs is known to have a low level of activity in the absence of Ku protein (33), the inhibition seen with the more effective RNAs probably reflects near-total ablation of Ku protein regulatory function.

The RNAs that failed to inhibit DNA-PK may bind to nonessential regions of the Ku protein. We note, however, that some of the inhibitory and noninhibitory RNAs share common conserved sequence motifs, which suggests that they recognize the same site in the Ku protein. In these cases, the failure to inhibit DNA-PK in a functional assay may be related to other properties of the RNA, for example, aggregation when prepared at high concentrations.

RNAs Cannot Activate DNA-PK Directly. We wished to determine whether any of the RNAs were capable of activating, as opposed to inhibiting, DNA-PK phosphorylation activity. None of the RNAs significantly increased DNA-PK activity in a standard assay performed in the presence of DNA. However, under these conditions, the DNA-PK was already highly active, and a weak ability of RNA to activate DNA-PK might have gone undetected. Indeed, displacement of DNA by a weakly activating RNA may have resulted in partial inhibition of DNA-PK activity.

To address this issue, we performed DNA-PK assays in the absence of DNA. Four RNAs were tested, including one that had given no effect on DNA-PK activity in the earlier assays (#52), two that had given partial inhibition (SC1 and SC9), and one that had given potent inhibition (SC4). None of these four RNAs showed any ability to activate DNA-PK above background levels in the absence of DNA (data not shown).

Competitive Binding Analysis between ds DNA and RNA. In principle, an RNA that binds to Ku protein could inhibit DNA-PK either by blocking the binding of Ku protein to

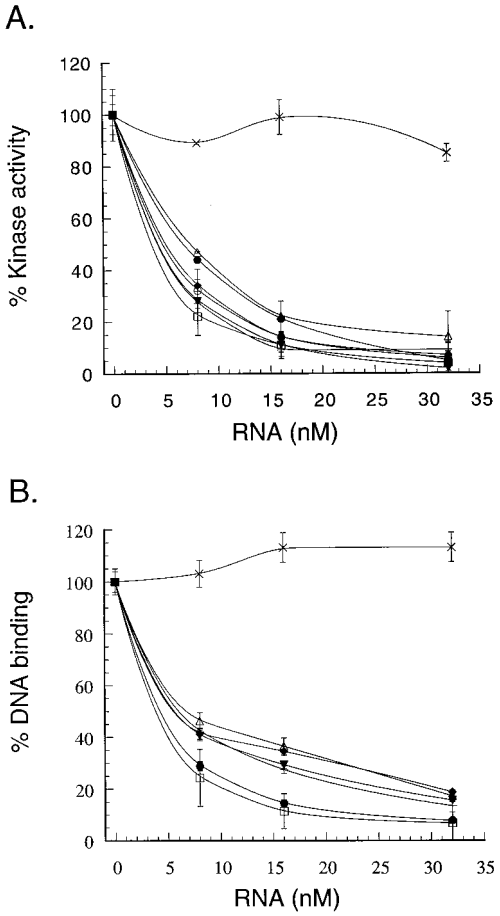

FIGURE 2: (A) Inhibition of the kinase activity of DNA-PK by aptamer RNAs. Each aptamer, as well as the unselected RNA pool, was tested in a standard DNA-PK assay as described in Experimental Procedures with the indicated RNAs: (×) unselected RNA; (○) #2; (□) #7-3; (△) SC4; (+) SC5; (◆) SC8; (▼) #1-2; (●) SC2. DNA-PK activity is expressed as a percentage of activity in the absence of RNA. Values shown are averages of duplicate reactions with standard deviations indicated. Background phosphorylation in the absence of DNA has been subtracted. (B) Competition of aptamer RNAs with double-stranded DNA for binding to Ku protein. Each aptamer, as well as the unselected RNA pool, was tested in a competitive binding assay. Purified Ku protein (2 nM) was incubated with radiolabeled 21 bp double-stranded DNA (1 nM) in the presence of various amounts of unselected RNA or aptamer. Symbols are the same as in panel A. A nitrocellulose filter binding assay was carried out as described in Experimental Procedures to measure Ku−DNA complexes. Binding is expressed as a percentage of Ku−DNA complexes detected in the absence of competitor. Values shown are averages of duplicate reactions with standard deviations indicated.

DNA or by blocking the interaction between Ku protein and DNA-PKcs. To distinguish these possibilities, we tested the RNAs for their effect on Ku protein−DNA binding.

In these experiments, purified Ku protein was incubated with a radiolabeled DNA oligonucleotide in the presence or absence of nonradiolabeled RNA. Ku protein−DNA complexes were trapped in a nitrocellulose filter binding assay. The seven RNAs that functioned as effective inhibitors of DNA-PK enzyme activity also proved to be effective inhibitors of Ku protein−DNA binding activity (Figure 2B). By contrast, RNA from the unselected pool had no effect on Ku protein−DNA interaction (Figure 2B). The results suggest that all of the inhibitory RNAs that were tested work

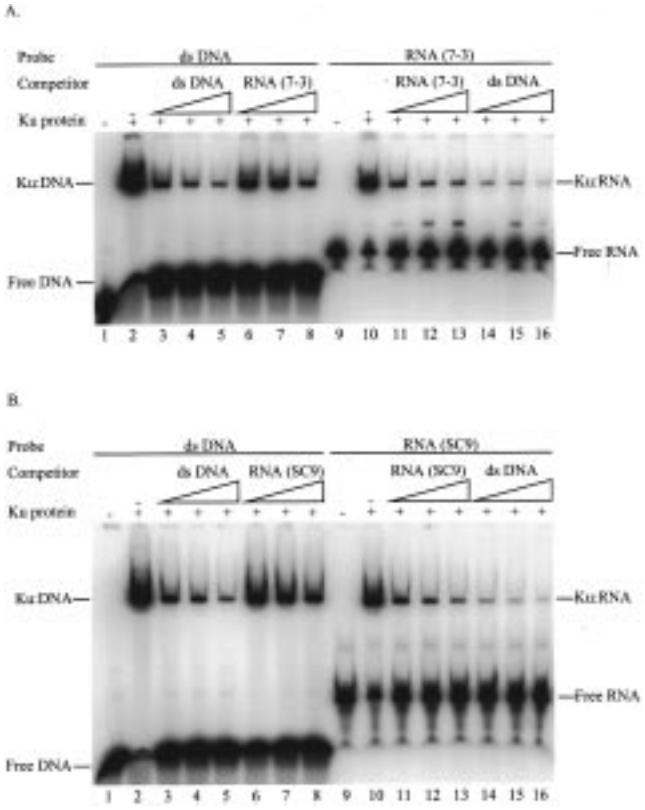

FIGURE 3: Cross-competition between RNA aptamers and DNA for Ku protein binding. Electrophoretic mobility shift assays were conducted with either radiolabeled 21 bp double-stranded DNA (1 nM) or aptamer RNAs (1 nM) as probes. (A) Lanes 1 and 9 show the migration of probes in the absence of Ku protein. Lanes 2−8 show Ku protein (2 nM) binding to a double-stranded DNA probe. Reactions in lanes 3−5 contain increasing amounts (8, 16, and 32 nM) of nonradiolabeled DNA as competitor. Reactions in lanes 6−8 contain increasing amounts (8, 16, and 32 nM) of nonradiolabeled aptamer RNA #7-3. Lanes 10−16 show Ku protein (2 nM) binding to radiolabeled RNA aptamer #7-3. Reactions contained various amounts of competitor DNA or RNA (8, 16, or 32 nM). (B) Binding competition assays as above using a different RNA aptamer (SC9).

primarily by interfering with protein−DNA interactions rather than protein−protein interactions.

Both of the subunits of Ku protein have a predicted net negative charge at neutral pH, and it may be that unfavorable electrostatic interactions restrict interaction with aptamer RNAs over much of the protein's surface. Given this constraint, it is perhaps not surprising that many of the aptamers are targeted toward the DNA binding site within the Ku protein. Although this site has not been fully mapped, it is likely that it contains basic residues that are capable of electrostatic interactions with the phosphate backbone of RNA and DNA.

To further explore the relationship between RNA and DNA binding sites in Ku protein, we performed electrophoretic mobility shift assays comparing radiolabeled RNA and DNA probes. Figure 3 shows that Ku protein forms stable complexes with both types of probes and that the complexes with RNA and DNA have similar electrophoretic mobilities. Both of the RNAs that were tested competed with DNA for binding to Ku protein (lanes 6−8 in Figure 3A). RNA #7-3 is a more effective competitor than SC9, consistent with the results of nitrocellulose filter binding assays (Figure 2 and data not shown). In the reciprocal experiment, nonradiola-

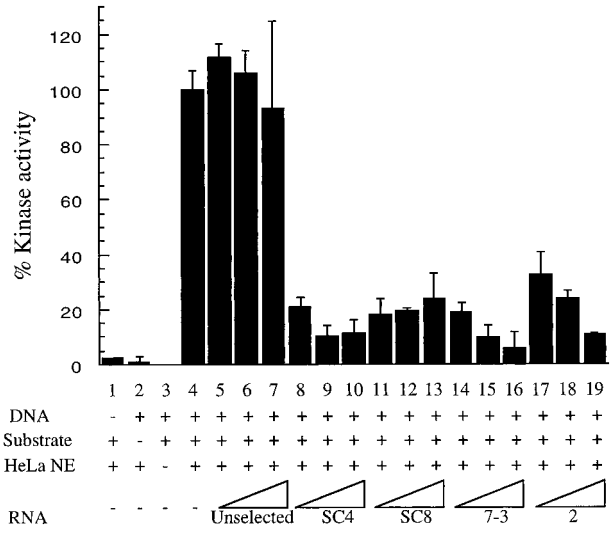

FIGURE 4: Inhibition of the kinase of DNA-PK by RNA aptamers in crude nuclear extract. Each of the indicated aptamers as well as the unselected RNA pool was tested for the ability to inhibit catalytic activity of DNA-PK in an unfractionated HeLa cell nuclear extract. Reactions contained 2 μL of HeLa cell nuclear extract (0.4 μg of protein), 5.3 nM DNA, and other components as in Figure 2. Reactions were performed in the presence of various amounts of aptamer RNA (250, 500, and 1000 nM) as indicated. DNA-PK activity is expressed as a percentage of activity in the absence of RNA. The background of radioactivity retained on the filter in the absence of nuclear extract has been subtracted. Values shown are averages of duplicate reactions with standard deviations as indicated.

beled DNA fragments competed with labeled RNA for binding to Ku protein (lanes 14−16 in Figure 3). As controls, DNA and each RNA were shown to effectively self-compete for binding to Ku protein (Figure 3, lanes 3−5 and 11−13), confirming the fidelity of the analysis.

Notably, neither the DNA nor the RNA competitors induced the formation of supershifted complexes. The absence of supershifted complexes provides additional evidence that RNA and DNA cannot bind simultaneously to Ku protein but rather bind competitively to the same site.

Specific Inhibition of DNA-PK by Aptamers in Crude Cell Extracts. Part of the rationale for identifying RNAs that interact with Ku protein was to develop potential tools, aptamers, for experimental modulation of DNA-PK activity. To be useful, such aptamers not only must recognize Ku protein but also must be able to do so in a complex milieu containing other macromolecules. Although the experiments in the preceding sections show that RNAs bind to Ku protein and inhibit the catalytic activity of purified DNA-PK, the degree to which the aptamer RNAs engage in unwanted interactions with other cellular components has not yet been addressed.

To determine whether the RNAs were specific for Ku protein, we measured the ability of the aptamers to inhibit DNA-PK in crude HeLa cell nuclear extracts. As shown in Figure 4, phosphorylation of the p53-derived peptide substrate by crude extracts is strongly stimulated by addition of double-stranded DNA. This suggests that DNA-PK is the major kinase that phosphorylates this substrate under the conditions used. Four different aptamer RNAs, all of which had previously been shown to inhibit purified DNA-PK, were tested in the crude extract. All four reduced enzyme activity to near-basal levels. RNA from the unselected pool had no

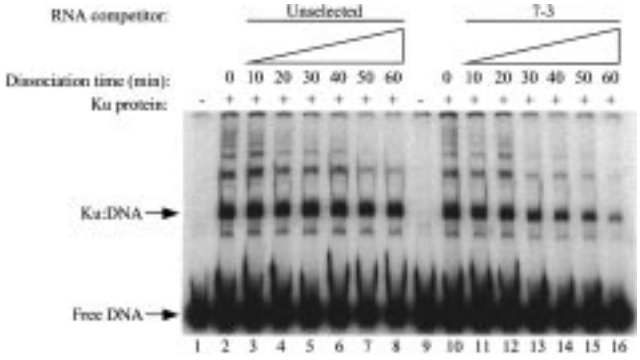

FIGURE 5: Dissociation of Ku−DNA complexes in the presence of the aptamer RNA. Purified Ku protein (0.32 nM) was incubated with a radiolabeled DNA fragment (0.38 nM) and complexes were allowed to form for 30 min at room temperature. In lanes 3−8 and 11−16, nonradiolabeled competitor RNAs (lanes 3−8, 100 nM unselected RNA; lanes 11−16, 100 nM RNA aptamer #7-3) were added, and incubation was continued for the times indicated. In lanes 2 and 10, no competitor was present. Lanes 1 and 9 show the migration of free probe in the absence of Ku protein.

effect at concentrations up to 1000 nM (Figure 4, lanes 5−7). These results show that the aptamers are sufficiently selective that they can recognize Ku protein and inhibit DNA-PK activity even in a crude preparation.

Dissociation of Ku−DNA Complexes by Aptamer RNAs. Previous work has suggested that once the Ku protein binds to double-stranded DNA, the resulting complexes are not readily disrupted by a second DNA added as competitor, except when the first and second DNAs have cohesive ends (*19*). To find out whether aptamers were capable of capturing Ku protein from preformed complexes, we performed an experiment in which a double-stranded DNA fragment was incubated with Ku protein for 30 min and then challenged with either aptamer RNA or RNA from the unselected pool. Incubation was continued for the times indicated in Figure 5 and the amount of Ku−DNA complex remaining was measured. Ku protein−DNA complexes steadily dissociated in the presence of aptamer #7-3 (Figure 5, lanes 11−16). In contrast, the amount of Ku protein−DNA complexes remained almost unchanged when challenged with unselected RNA (Figure 5, lanes 3−8). This result demonstrates that the aptamer RNA can compete with double-stranded DNA for the binding of Ku protein even if the aptamer RNA is added after Ku−DNA complexes have formed.

DISCUSSION

We have shown that Ku protein binds to small RNAs, that this binding is specific, and that it is comparable in strength to the binding to DNA. Many of the RNAs that we isolated bound competitively with DNA and consequently blocked Ku protein function in a DNA-PK phosphorylation assay. None of the RNAs that were tested activated DNA-PK directly, in the absence of DNA, suggesting that occupancy of the Ku protein DNA binding site by a nucleic acid is not, in itself, sufficient to stimulate DNA-PK enzymatic activity.

We identified 19 different Ku-binding RNAs from a pool that originally contained about 10^{14} different RNAs. The sequences of the selected RNAs appear highly nonrandom. There is an 11-base motif that is strikingly conserved in seven of the aptamers. These and other conserved sequences are

presumably involved in base-specific contacts with Ku protein. In addition to base-specific interactions, it is likely that secondary structure and tertiary structure of the aptamers play a crucial role in Ku protein recognition. We have applied the energy-based method of Zuker to predict secondary structure of the aptamers (*35*, *36*). Two of the aptamers with the most extensive conserved sequence motifs (SC4 and #2) are predicted to adopt similar, stable secondary structures with the conserved motifs present as single-stranded loops (not shown). These predictions provide a starting point for more systematic investigation of the structural features of these RNAs.

We have not directly investigated whether free RNA ends are required for Ku protein binding. Although free ends can be sites for binding of Ku protein to DNA, studies with various artificial DNA constructs have shown that Ku protein can bind at any region of single- to double-strand transition, including nicks, gaps, hairpins, and bubbles. Since single- to double-strand transitions are common in RNA secondary structure, we expect that RNA ends, as such, should not be required for Ku protein binding. Thus, we expect that the aptamer sequences should be capable of binding Ku protein when embedded in a longer sequence context or even when present in a circular RNA.

The existence of RNAs that bind tightly to Ku protein is consistent with suggestions that Ku protein function may be regulated to some extent by RNA in the normal cell (*29*). One site where such interactions might occur is in the nucleolus. DNA-PK and Ku protein regulate RNA polymerase I, which is localized in the nucleolus (*39*−*41*). Immunofluorescence studies show that Ku protein is present in the nucleolus, that the degree of nucleolar localization is cell-cycle-dependent (*28*), and that nucleolar localization is RNase-sensitive (*29*). It may be fruitful to investigate whether Ku protein associates with specific nucleolar RNAs and whether this is connected with the role of Ku protein in regulating RNA polymerase I activity.

Another implication of our present studies is that aptamers might be useful as tools for investigation of Ku protein function in cells and cell extracts. Although a number of antibodies to Ku protein have been described, aptamers have different and complementary properties. We have screened a variety of commercially available monoclonal antibodies (N3H10, N9C1, 111, 162, and S5C11) and found that all of these produced supershifts in an electrophoretic mobility shift assay, indicating that they recognize epitopes outside the DNA binding site (unpublished results). By contrast, the aptamers competitively inhibited DNA binding, indicating that they are targeted directly to the DNA binding site of Ku protein (Figures 2 and 3). This property makes the aptamers useful for probing the role of Ku protein−DNA interactions in various physiological processes. Although DNA-PK is clearly involved in DNA repair and recombination, it has also been proposed that it is involved in a number of other processes, including the stress response, viral infection, and transcriptional regulation (reviewed in ref *42*). Several strategies have been described for targeted expression of small RNAs in subnuclear compartments (*43*−*45*). An ability to express inhibitory aptamers in vivo may provide a way to further investigate the physiological role of Ku protein.

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