Ku is involved in the metabolism of DNA ends, DNA repair, and the maintenance of telomeres. It consists of a heterodimer of 70- and 80-kDa subunits. Recently we have demonstrated that Ku70 interacted with TRF2, a mammalian telomere-binding protein. Using the same yeast two-hybrid screening system, we now show that Ku70 also interacts with heterochromatin protein 1α (HP1α), a protein known to be associated with telomeres as well as heterochromatin. HP1 is a suppressor of the position effect variegation in Drosophila and acts as a transcriptional suppressor in mammalian cells. The interaction with Ku70 in the two-hybrid system was confirmed by a glutathione S-transferase pull-down study using bacterial recombinant proteins in vitro. The interaction was also reproduced in vivo in HeLa cells, where endogenous Ku70 coimmunoprecipitated with HP1α. This interaction was more effective in acidic pH and weakened considerably as the pH of the reaction buffer was elevated up to 7.5. Ku80 did not interact with HP1α directly. The interaction domains of Ku70 and HP1α included the Leu-Ser repeat (amino acids 200–385) and the chromo shadow domain, respectively. Ku70 was largely colocalized with transfected HP1α but not with a C-terminal deletion mutant, HP1αΔC. In contrast to HP1α, Ku70 did not repress transcriptional activity of the reporter gene when tethered to DNA after transfection to mammalian cells. The implication of this interaction is discussed.

Ku was originally identified as a major autoantigen in patients with autoimmune diseases such as scleroderma-polymyositis (1). Ku is a heterodimer of 70- and 80-kDa subunits (2, 3) and binds to DNA ends, nicks, or single- to double-strand transition (2, 3). Ku binds to the DNA-dependent protein kinase catalytic subunit of DNA-protein kinase complex, which has been shown to be crucial for nonhomologous double-strand break DNA repair and V(D)J recombination (4–10).

It has been shown that Ku played an important role in the regulation and maintenance of telomeres as well. In Saccharomyces cerevisiae, mutants of either human Ku70 or Ku80 homologues, YKU70/HDF1 and YKU80/HDF2, were shown to have abnormally short telomeres (11, 12) and disrupted subnuclear organization of telomeres (13). Mutations in either Ku subunit led to enhanced instability of telomeres by increasing their sensitivity to either degradation or recombination reactions (14). Ku has been shown to be an integral component of yeast telomeres (15) and to bind human telomeric DNA in vitro (16). Ku was also shown to be associated with mammalian telomeres (17).

Ku is a relatively abundant protein and is present much more than the DNA-dependent protein kinase catalytic subunit in the nuclei (5, 18). Recently, we showed that Ku was present in the nuclear matrix as well as in the soluble fractions of human nucleus (19). In the nuclear matrix, Ku70 appeared to be present in a greater amount than Ku80 (19). Thus, it appeared that Ku70 might bind other proteins to perform diverse biological functions at least in certain cellular compartments. Such a possibility prompted us to look for additional interacting proteins of Ku70.

A number of attempts have been made to identify proteins that interact with Ku70 using the yeast two-hybrid system. Human Ku70 was identified to be an interacting protein of GCN5/CREB/TAF1250 acetyltransferase (20), oncoprotein p85α (21), and apolipoprotein J (22). However, the search has been limited by the predominant interaction with Ku80 in the yeast two-hybrid system (23, 24). To avoid such a problem, we screened a HeLa cell library using a partial Ku70 cDNA bait, which did not overlap significantly with the proposed binding sites to Ku80 (23–25). Using the system, we have shown that Ku70 interacts with TRF2, a telomere-binding protein (26).

Here, we show that Ku70 interacts with HP1α, which is one of the three mammalian HP1 family proteins. HP1 homologues have been found in many other organisms from Schizosaccharomyces pombe (27) to mammals (28, 29). HP1 is a nonhistone chromosomal protein suppressor of position effect variegation in Drosophila (30, 31). It is associated with the heterochromatin region (30, 31) and telomeres (32), and prevents telomere fusion (32). It is of interest that the yeast Ku70 homologue Hdf1 has been shown to interact with a telomere-binding protein, Sir4, which is also a suppressor of the telomere position effect, in S. cerevisiae (33).

**EXPERIMENTAL PROCEDURES**

Plasmid Construction for Yeast Two-hybrid Assay—All constructions were done according to standard methods and verified by sequencing. For the yeast two-hybrid analysis, we used the system developed by the Brent laboratory (34) that used LexA as the DNA-binding protein, B42 as a transcriptional activator, and lacZ and LEU2 as reporters. The full-length human Ku70 and Ku80 cDNA were kind gifts from Dr. W. H. Reeves (35). A region corresponding to the amino acid residues 200–385 (186 aa) of Ku70 was fused to the LexA DNA binding domain (LexA-Ku70/186) and used as bait in the yeast two-hybrid screening. For control baits, LexA-Ku70/139, LexA-Ku80/185, LexA-DDX3, and LexA-
thyroid receptor were generated. LexA-Ku70/139 contained residues 471–609 (139 aa) of Ku70 (35). LexA-Ku80/185 contained the N-terminal 185 amino acid residues of Ku80 (36). LexA-DDX3 contained the full-length human DDX3 (37). LexA-thyroid receptor was described previously (38). DNA fragments were amplified by polymerase chain reaction (PCR) and cloned into the pET28 or pGEX-4T-1 plasmid vectors. Other plasmids were constructed similarly for the bait-prey interaction experiments.

To further delineate the interaction domains of Ku70, two truncated versions of the original bait, LexA-Ku70/44 and LexA-Ku70/119, were constructed. LexA-ku70/44 included amino acid residues 200–243 (44 aa), which represented the Leu-See repeat (39). LexA-ku70/119 included amino acid residues 267–385 (119 aa), which corresponded to the flanking anionic domain. To delineate interacting domains of the selected H1bp/E11, three truncated versions of H1bp were cloned into the prey vector pJG4-5: H1bp/N (containing aa 1–58), H1bp/M (57–157), and H1bp/C (111–191). Deletion mutants were created by using convenient restriction sites and/or by PCR with appropriate primer pair combinations and verified by sequencing. The plasmids were introduced into EGY48-expressing LexA-Ku70/188 and/or LexA-Ku70/119 hybrid proteins.

**Yeast Two-hybrid Screen**—The baits were introduced into yeast S. cerevisiae strain EGY48 (Ma7a, his3, trp1, ura3-52, leu2::pLeu2-LexAop-hisG/P5H18-34 [LexAop-leu2 reporter]) by a modified lithium acetate method. The transformants were selected on the yeast synthetic medium (Ura−, His+, and Leu−) and used as a host for transformation with HeLa cDNA library as described previously (38). The cDNA fragments were cloned in pJG4-5 using EcoRI and XhoI to generate B24 fusion proteins, and the expression of the fusion protein was designed to be induced by the presence of galactose. The cDNA was introduced into the competent yeast cells, and transformants were selected for tetracycline prototrophy (plasmid marker) on the synthetic medium (Ura−, His+, and Trp−) containing 2% galactose. All transformants were pooled and resuspended on the synthetic medium (Ura−, His+, Trp−, and Leu−) containing 2% galactose to induce the introduced cDNA. Cells growing on the selection media were tested on the synthetic medium (Ura−, His+, Trp−, and Leu−) containing 2% galactose (inducing condition) and 2% glucose (noninducing condition) to confirm the dependence of their growth on the presence of galactose. Cells growing only on the galactose media were selected and streaked on the synthetic medium (Ura−, His+, Trp−, and Leu−) containing 2% galactose or 2% glucose with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside to test the β-galactosidase activity. The cells expressing both reporter genes only in the presence of galactose were finally isolated to confirm the interaction between the bait and prey vector.

Production of Recombinant Ku70 Proteins in Escherichia coli—Three in-frame fusions of Ku70 were constructed with the pET28 vector (Novagen) as N-terminal (His)9-tagged proteins for bacteriolar expression. In-frame fusion of the LexA-ku70/188 bait was constructed by cloning the segment from 575 bp from pEG202/Ku70/188 into the EcoRI site of pET28a. After transformation to E. coli strain DE3, the recombinant protein turned out to be insoluble.

In-frame fusion of full-length Ku70 was constructed by ligating first the BamHI-HindIII fragment of 1476 bp from pET28a/Ku70/1–482 and then the HindIII-XhoI fragment of 468 bp from pET28a/Ku70/176–609, followed by cloning into the BamHI-XhoI sites of pET28a. When the recombinant full-length Ku70 was expressed in bacteria, its N terminus was cleaved. Thus, it was necessary to cleave off the N-terminal part of Ku70.

To construct a plasmid expressing the N-terminal deleted Ku70 gene product, Ku70N, pET28a/Ku70/200–690, a fragment of 1233 bp encompassing amino acids 200–609, was obtained by PCR using a sense primer (5′-TCACTTGGAGTGGTACCTTG-3′) and an antisense primer (5′-TCAGTGGATGCTCTAAGGC-3′). The PCR product was first cloned into a vector, pCR2.1 (Invitrogen), and then a 1250-bp EcoRI fragment from pCR2.1-Ku70/410 was cloned into the EcoRI site of pET28a. The Ku70N fusion protein, which had amino acid residues 200–609 and 38 additional amino acids, was partly soluble. The fusion proteins were purified using a His-bind resin column according to the manufacturer’s instructions (Novagen). They were used to immunize rabbits.

**Production of Recombinant HP1a Proteins in E. coli**—In-frame fusions of full-length HP1a (aa 1a–191), HP1aSN (aa 111–191), and HP1aAC (aa 1–137) were similarly cloned in pET28 as well as pGEX-4T-1 vectors (Amersham Pharmacia Biotech) for GST tagging. To construct a plasmid expressing intact HP1a, a full-length cDNA was generated by EcoRI and XhoI liberation from the library prey vector E11, followed by cloning into the EcoRI-XhoI sites of pET28 as well as pGEX-4T-1 vectors. HP1aSN and HP1aAC were constructed by a similar procedure, but the inserts were obtained by PCR with a full-length cDNA clone, E11, using appropriate primer combinations. The PCR products were digested with EcoRI and XhoI followed by cloning into the EcoRI-XhoI sites of pGEX-4T-1 vector that had been predigested with the same enzymes.

**GST Pull-down Assay**—GST-HP1a, GST-HP1aSN, and GST-HP1aAC fusion proteins were expressed in E. coli and purified on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). For a negative control, GST without fusion was expressed and purified similarly. GST proteins were applied to glutathione-Sepharose 4B beads, which were eluted from the beads using the elution buffer (10 mM glutathione, 50 mM Tris, pH 8.0) according to the manufacturer’s instructions. To 10 µg of GST-HP1a and comparable amounts of other GST fusion proteins, equimolar purified His-tagged Ku70N was added in the reaction buffer (10 mM 1,4-piperazinediethanesulfonic acid, pH 6.5, 0.5% Nonidet P-40, 83 mM KCl, 17 mM NaCl, 1 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 1 mM dithiothreitol) containing 3 µg urea. After 1 h incubation, the mixtures were dialyzed overnight at 4 °C in the reaction buffer without urea. Then glutathione-Sepharose 4B beads were added and incubated for 4 h. Beads were washed five times in the reaction buffer and resuspended in Laemmli buffer, and the proteins were separated on 10% SDS-polyacrylamide gel electrophoresis. Proteins were blotted onto a nitrocellulose membrane and probed with mouse monoclonal anti-H7.tag antibody (Novagen), followed by goat anti-mouse as a second antibody (Sigma). Antibodies were visualized by using a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color reaction (Roche Molecular Biochemicals). To figure out the influence of pH on the interaction, GST pull-down experiments were repeated in the reaction buffer at pH 6.2, 6.5, 6.8, 7.2, and 7.5, respectively.

**Commmunoprecipitation of HP1a and Ku70 in Vivo**—For whole-cell extracts, 5 × 109 HeLa cells were washed with cold phosphate-buffered saline, harvested by scraping, and centrifuged for 2 min in an Ependen microfuge at 4000 × g. The cells were resuspended in 200 µl of reaction buffer and Dounced on ice thoroughly. Immunoprecipitation was performed with anti-HP1a antibody. The affinity-purified anti-HP1a antibody was obtained from anti sera of a rabbit, which was immunized using the purified GST-HP1a fusion protein as described previously (19). For a negative control, nonimmune rabbit serum was used at the same concentration. Immune complexes were precipitated with protein A-Sepharose beads. The beads were washed three times in PBS, resolved in the SDS-polyacrylamide gel electrophoresis sample buffer, and electrotransferred to a nitrocellulose membrane. Communoprecipitation of Ku proteins was analyzed by immunoblotting with isoforms of anti-Ku70 and anti-Ku80 antibodies, respectively (19). Immune complexes were visualized using a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color reaction and/or the enhanced chemiluminescence method (ECL detection system; Amersham Pharmacia Biotech).

**Immunofluorescence Microscopy of Transfected Cells**—COS cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml streptomycin, and 100 units/ml penicillin at 37 °C in 5% CO2. The HP1a and HP1aAC DNAs with T7.tag were cloned into pcDNA3 (Invitrogen), a mammalian expression plasmid that contains a cytomegalovirus promoter. COS cells were transfected with 10 µg of plasmid DNA/30-mm dish using lipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Transfected COS cells were grown on coverslips.

At 48 h after transfection, coverslips were rinsed with PBS and fixed in cold methanol (−20 °C) for 10 min. Then primary antibodies were incubated with cells for 2 h at room temperature. Rabbit polyclonal anti-H7.tag and monoclonal anti-Ku70 antibodies were described above. Mouse polyclonal anti-H7.tag antibody was a generous gift from Dr. William C. Earnshaw (University of Edinburgh, Edinburgh, United Kingdom). Monoclonal anti-T7.tag antibody (Novagen) and goat anti-Ku70 antisera (Santa Cruz Biotechnology) were purchased. For datasource, Texas red-labeled anti-mouse and fluorescein isothiocyanate-labeled anti-goat secondary antibodies (Sigma) were applied for 1 h in 1% bovine serum albumin (BSA) in PBS (Novagen) with 4,6-diamidino-2-phenylindole (1 µg/ml PBS) for 15 s (Sigma). Cells were viewed in a confocal laser microscope (Zeiss LSM510). As controls, cells were stained with primary or secondary antibody alone. Control slides did not display significant fluorescence in any case.

**Transfections and Reporter Assay**—pM1 and pM1-H7.tag effector plasmids and G5-SV40-CAT reporter plasmid were generous gifts from...
**RESULTS**

**Identification of HP1α as a Binding Partner of Ku70**—cDNA corresponding to the amino acid residues 200–385 (186 aa) of Ku70 was fused to the LexA DNA binding domain (LexA-Ku70/186) and used as bait in the yeast two-hybrid screening. This region included the periodic repeat of leucine and serine (aa 215–243) in every seventh position and an anionic domain (35). It was chosen as bait because (i) it does not overlap significantly with the published Ku80 binding sites, and (ii) it contains a domain that is reminiscent of a leucine zipper (42) and therefore likely to interact with other molecules.

Using the LexA-Ku70/186 bait, a HeLa cell cDNA library was screened as described under “Experimental Procedures.” The bait did not have any intrinsic activity of transcriptional activation for the reporters. Approximately $3 \times 10^5$ independent transformants were plated and reseeded in the selection media (Ura-, His-, Trp-., and Leu-) containing 2% galactose to induce the expression of cDNAs. In the selection media, a total of 72 colonies showed galactose dependence. The plasmids were extracted by yeast miniprep, and the cDNAs were PCR-amplified with primers derived from the vector pJG4–5, followed by sequence determination.

DNA sequencing and data base searches revealed that the nucleotide sequence of nine clones encoded human HP1α (32).

Although 8 clones encoded the full-length HP1α, one contained a 5’-truncated cDNA encoding amino acids 32–191 of HP1α. Thus, the binding site to Ku70 did not appear to involve the N terminus.

To confirm the specificity of the interaction, three additional baits containing similar leucine zipper-like domains were tested as well; they included LexA-Ku70/139, LexA-Ku80/185, and LexA-DDX3/260 (Fig. 1). LexA-Ku70/139 encompassed amino acid residues 471–609 of Ku70, which included the second leucine zipper-like domain. LexA-Ku80/185 and LexA-DDX3/260 included the leucine zipper-like domains of Ku80 and the putative RNA helicase DDX3, respectively. As shown in Fig. 1, HP1α did not interact with any one of the control baits, indicating that the association of Ku70/HP1α was not mediated by nonspecific interaction of the leucine zipper-like α-helix domains.

**Interaction Sites of Ku70 and HP1α**—To map the interaction domains of the Ku70 with HP1α, three deletion derivatives of HP1α, its three deletion derivatives, were generated: HP1α/C (aa 111–191), HP1α/N (contain- ing aa 1–58), and HP1α/H (aa 57–137), and HP1α/C (aa 111–191), representing the chromo domain (43), “hinge” region (44), and chromo shadow domain (45) of HP1α, respectively. Mutant forms of HP1α were tested for their ability to bind to the LexA-Ku70/186 bait by yeast two-hybrid assay. As shown in Fig. 1, HP1α/C was solely responsible for the interaction with Ku70. Thus, it appeared that the chromo shadow domain, which spanned amino acid residues 123–175, was the Ku70 binding site of HP1α.

**GST Pull-down Analysis**—To validate the results obtained with the yeast two-hybrid system, the interaction between recombinant Ku70 and HP1α proteins was studied in vitro. For the GST pull-down experiments, GST-HP1α, GST-HP1α

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**FIG. 1. Yeast two-hybrid analysis.** Domains of Ku70 are shown at the top of the **BAIT** section. Bars below the **lines** designate inserts of three baits used for the yeast two-hybrid analysis. Inserts of the Ku80 and DDX3 control baits are shown below. Domains of HP1α are shown at the top of the **PREY** section. C11 was one of the prey clones containing full-length HP1α. The full-length HP1α and HP1α/C (aa 111–191) interacted with the bait Lex-Ku70/186, confirming that the interaction site was in the chromo shadow domain. The interactions were confirmed by reciprocal experiments. CD, chromo domain; CSD, chromo shadow domain.
and GST-HP1αSc fusion proteins were expressed in E. coli and purified on a glutathione-Sepharose 4B column. As a binding partner, recombinant His-tagged Ku70ΔN was expressed and purified on His binding resin (see “Experimental Procedures”). To increase the solubility of recombinant His-tagged Ku70ΔN, 3 M urea was added to the reaction buffer. After the incubation, the mixtures were dialyzed in the reaction buffer without urea. Recombinant HP1α proteins were pulled down by glutathione-Sepharose 4B beads, and the proteins were resolved on 10% SDS-polyacrylamide gel electrophoresis. The immunoblotting using anti-T7.Tag antibody, Ku70 was shown to be co-precipitated with GST-HP1α and GST-HP1αSc but not with GST-HP1αN or GST alone. This result confirmed that the binding site was in the C-terminal region of HP1α, the chromo shadow domain. B, pH effect on the interaction in vitro. GST pull-down experiments were done in the reaction buffer at various pH: 6.2, 6.5, 6.8, 7.2, and 7.5, respectively. Ten μg of purified Ku70ΔN was incubated with 5 μg of purified GST-HP1α in every experiment. By immunoblotting using anti-T7.tag antibody, Ku70ΔN was shown to be most effectively pulled down at pH 6.2 and 6.5 through the interaction with GST-HP1α. The pulled down Ku70ΔN decreased gradually as the pH was elevated until it was detected barely at pH 7.5. MW, molecular weight. WB, Western blot.

**Fig. 2. GST pull-down experiment using recombinant Ku70 and HP1α.** A, ten μg of purified His-tagged recombinant Ku70ΔN was incubated with equimolar GST-HP1α, GST-HP1αSc, or GST-HP1αN fusion proteins in the reaction buffer (10 mM 1,4-piperazinediethanesulfonic acid, pH 6.5, 0.5% Nonidet P-40, 83 mM KCl, 17 mM NaCl, 1 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 1 mM dithiothreitol) containing 3 μM urea to increase the solubility of Ku70ΔN. Then the mixtures were dialyzed overnight at 4 °C in the reaction buffer without urea. Recombinant HP1α proteins were pulled down by glutathione-Sepharose 4B beads, and the proteins were resolved on 10% SDS-polyacrylamide gel electrophoresis. The immunoblotting using anti-T7.tag antibody, Ku70 was shown to be co-precipitated with GST-HP1α and GST-HP1αSc, whereas no interaction was noted with GST-HP1αN. By immunoblotting, it was confirmed to be Ku70 (B). MW, molecular weight; Ig, immunoglobulin heavy chain; WB, Western blot.

**Fig. 3. In vivo interaction of Ku70 and HP1α.** The HeLa cell extract was incubated with affinity-purified anti-HP1α antibody. For a negative control, preimmune rabbit serum was applied at the same concentration. Immune complexes were precipitated with protein A-Sepharose beads, and the proteins were resolved in SDS-polyacrylamide gel electrophoresis. By Coomassie blue staining, a band of ~70-kDa protein was shown to co-precipitate with HP1α (A). By immunoblotting using monoclonal anti-Ku70 antibody, it was confirmed to be Ku70 (B). MW, molecular weight; Ig, immunoglobulin heavy chain; WB, Western blot.

**Analysis of Transcriptional Repressor Activity—**It has been shown that HP1α had transcriptional repressor activity in mammalian cells (40, 41). Thus, we decided to determine whether Ku70 had a transcriptional repressor activity. A fusion construct of Ku70 with the GAL4-DBD was generated for amounts of purified recombinant His-tagged Ku70ΔN and GST-HP1α, Ku70ΔN was most efficiently pulled down by GST-HP1α at pH 6.2, and then the interaction weakened gradually as the pH was elevated to 7.5 (Fig. 2B).

**Ku70 Colocalized with HP1α but Not with HP1αSc—**To confirm the interaction between Ku70 and HP1α in vivo, we examined the subcellular localization of wild-type HP1α and its deletion derivative, HP1αSc, after transfection into COS cells. On immunostaining using anti-T7.Tag antibody, ~5% of cells displayed the expression of transfected HP1α as a punctuated pattern throughout the nuclei (Fig. 4A). No cytoplasmic staining was present. Rabbit and mouse anti-HP1α antisera displayed a similar distribution and immunostaining pattern of positive cells. By double immunofluorescence microscopy, the immunostaining pattern of Ku70 did not appear to change considerably in those cells (Ref. 19 and Fig. 4B). Transfected HP1α and native Ku70 largely colocalized with minor exceptions (Fig. 4, A–C).

Transfected HP1αSc was detected in <1% of total cells. Although the nuclei were immunostained primarily, it was also detected as small granules in the perinuclear cytoplasm in some cells. In the nuclei, HP1αSc was mostly detected as irregular nodules, which tended to be much larger than the spots of wild-type HP1α (Fig. 4D). By double immunofluorescence staining, HP1αSc and Ku70 were present in a mutually exclusive manner (Fig. 4, D–F). The result was consistent with the yeast two-hybrid and biochemical data indicating that the binding site of HP1α to Ku70 was at the C-terminal region.
transfection into COS cells together with a CAT reporter plasmid bearing five GAL4 binding sites upstream of the SV40 promoter. As reported previously (40, 41), the expression of HP1α-GAL4-DBD fusions resulted in a 25–50-fold decrease in the activity from the SV40 reporter, compared with the control obtained with the GAL4-DBD alone (Fig. 5). However, the expression of Ku70-GAL4-DBD or Ku70Δα-GAL4-DBD fusions did not show any evidence of transcriptional repression but a slight increase of the activity from the SV40 reporter (Fig. 5).

**DISCUSSION**

**Interaction of Ku70 and HP1α**—The growing list of interacting proteins of Ku and HP1 indicates that they are involved in diverse biological functions. HP1 interacts with a number of proteins involved in gene silencing: suppressors of variegation 3–7 (46), suppressors of variegation 3–9 (47), and transcriptional intermediary factor α and β (48, 49). It also interacts with other nuclear proteins, including lamin B receptor (50), inner centromere protein (44), chromatin assembly factor 1 (51), SP100, a major component of promyelocytic leukemia protein nuclear bodies (40, 41), origin recognition complexes 1 and 2 (52), and ATR-X syndrome, a putative helicase and transcriptional regulator (53). So far, no protein has been shown to interact with both Ku and HP1α. Ku and HP1α may perform diverse biological functions depending on which binding partner they interact with in the given nuclear microenvironment. Because Ku80 did not interact with HP1α, it is suggested that subunits of Ku might function independently from each other depending on binding partners.

**Chromo Shadow Domain of HP1α as Interaction Site**—HP1α has a chromo domain, chromo shadow domain, and hinge region (43–45). Among the interacting proteins, origin recognition complex and inner centromere protein bind to the chromo domain (52) and hinge region (44) of HP1α, respectively. Except for the two, all others interact with HP1α through the chromo shadow domain. Our data show that Ku70 also binds to the chromo shadow domain of HP1α. Taken together, it appears that the chromo shadow domain is a major site for interaction with other nuclear proteins, which would be critical for the function of HP1α.

**Fig. 4. Immunofluorescence microscopy of transfected COS cells.** COS cells were transfected with either pcDNA3/HP1α or pcDNA3/HP1αΔα using lipofectAMINE reagent (Life Technologies). After 24 h, cells were fixed in cold methanol, and then double immunostaining was done using monoclonal anti-T7.Tag antibody and polyclonal anti-Ku70 antibody. Transfected HP1α was expressed as a punctated pattern in ∼5% of cells (A; rhodamine-labeled). Native Ku70 distributed similarly in the cells (B; fluorescein isothiocyanate-labeled). A merged image showed that Ku70 and transfected HP1α largely colocalized except for a few spots (C). Transfected HP1αΔα was detected in ∼1% of cells. HP1αΔα was mostly detected as nodular aggregates (D), which tended to be much larger than the spots of full-length HP1α (A). By double immunostaining, HP1αΔα and Ku70 were present in a mutually exclusive manner (E and F). Slides were examined using a Zeiss LSM510 confocal microscope.

**Fig. 5. Transcriptional regulator activity of HP1α and Ku70 in transfected mammalian cells.** The diagram at the top shows the reporter plasmid, which has five GAL4 binding sites. The mammalian expression vector pM1 contains the DNA binding domain of GAL4 under the control of the early SV40 enhancer. One μg of pM1/HP1α, pM1/Ku70, or pM1/Ku70Δα (aa 200–609) plasmid was transfected to HeLa cells together with the G5-SV40-CAT reporter plasmid using lipofectAMINE reagent (Life Technologies). At 48 h after transfection, the cell lysates were analyzed for CAT activity (Promega). The CAT reporter activity was normalized for transfection efficiency to an internal β-galactosidase control and expressed as a percentage of the activity obtained with the vector alone. Every construct was tested in four different transfection experiments.

The LexA-Ku70/186 bait contained the Leu-Ser repeat and flanking anionic domain. Other leucine zipper-like domains of control proteins and that of Ku70 (aa 483–518) failed to interact with HP1α in our yeast two-hybrid system, confirming the specificity of the interaction. Further direct delineation of the interaction site was not possible, because the Leu-Ser repeat alone showed an intrinsic transcriptional activity, and the anionic domain alone did not interact with HP1α. Recently, a pentamer sequence, PXVXL, has been proposed to be a consensus sequence of the HP1α binding site (54, 55). Intriguingly, a compatible pentamer sequence, PLVLL (aa 352–356), was included in LexA-Ku70/119, representing the anionic domain. It is possible that the truncated version of Ku70 in LexA-Ku70/
119 is not in proper configuration for the interaction in the yeast two-hybrid system.

Transfection and Reporter Assay—In our transfection system, HP1a suppressed the transcriptional activity of the reporter as much as was reported previously (40, 41). However, Ku70 did not show any transcriptional suppressor activity when tethered to DNA in either full-length or N terminus-deleted form. Rather, the reporter transcriptional activity was increased slightly. This result suggests that certain transcriptional activators compete with HP1a for binding with Ku70 in the system. Because Ku itself has also been shown to repress glucocorticoid-induced mouse mammary tumor virus transcription (56) and glycoporphin B transcription mildly (57), the slight but consistent increase of the reporter transcriptional activity is in favor of the possibility of a competition.

pH Dependence of the Interaction May Represent a Control Mechanism—We have reported that Ku interacts with another telomere-binding protein, TRF2 (26), which has been shown to prevent end-to-end fusion of telomeres (58). Because Ku, TRF2, and HP1a share the implicated function of telomere maintenance, it appears that they perform the function in close collaboration with each other through direct interactions. Because TRF2 and HP1a interact with Ku70 through the same binding area, they may not be able to bind Ku70 simultaneously. In contrast to the interaction between Ku and TRF2, which works effectively in both acidic and basic environments (26), HP1a interacts with Ku70 in acidic conditions primarily. Thus, it is possible that the complex interaction may be controlled in vivo in such a way that the pH of the microenvironment in the nucleus would play an important role. Further study would be necessary to address the possibility.

HP1a as a Mammalian Counterpart of Yeast Sir Protein—Yeast genetic studies have shown that Ku is a key player in the replication and protection of telomeres (11–15). In addition, Mre11, Rad50, Xrs2, Rap1, Sir2, Sir3, Sir4, Rif1, and Rif2 are also required to maintain the telomeres normally (for review, see Ref. 59). Mutants of Rad50, Mre11, and Xrs2, which are known to form a protein complex, all display a telomere-shortening phenotype similar to that of Ku mutants (60, 61); however, in contrast to Sir proteins, the mutants are not defective in telomere silencing (60). Thus, the yeast telomere proteins may be divided into two groups, namely proteins with or without a silencing effect.

The specific interaction of HP1a with Ku70 suggests that HP1a is a mammalian counterpart of Sir4, which has been sought for. If HP1a represented a telomere protein of the silencing group, other telomere proteins such as TRF1, TRF2, TRF1-interacting nuclear protein 2 (62), and hRap1 (63) could be regarded as mammalian proteins that regulate the telomere length without a silencing effect. Another telomere protein, tankyrase, may regulate other proteins by ADP-ribosylation (64). Through the direct interaction with TRF2 and HP1a, Ku appears to play a key role in the maintenance and regulation of telomeres in mammalian cells as in yeast cells. The role of Ku70 in heterochromatin formation and maintenance remains to be elucidated.

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