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Molecular Shape and ATP Binding Activity of Rat p50, a Putative Mammalian Homologue of RuvB DNA Helicase[1]

Noriko Kikuchi,* Takashi Gohshi,* Shigeru Kawahire,† Taro Tachibana,‡ Yoshihiro Yoneda,‡ Toshiaki Isobe,§ Chun Ren Lim,‖ Kenji Kohno,‖ Tohru Ichimura,* Saburo Omata,* and Tsuneyoshi Horigome*,[2]

Department of Biochemistry, Faculty of Science, and †Course of Biosystem Science, Graduate School of Science and Technology, Niigata University, 2-Igarashi, Niigata 950-2181; ‡Department of Anatomy and Cell Biology, Osaka University Medical School, Yamadaoka, Suita 565-0871; §Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Minami-oosawa 1-1, Hachioji, Tokyo 192-0364; and ‖Laboratory of Animal Cell Function, Research and Education Center for Genetic Information, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101

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Based on partial amino acid sequences of p50 purified from a high-salt buffer extract of a rat liver nuclear matrix fraction, p50 cDNA was cloned and sequenced, and its amino acid sequence was predicted. The sequence contained helicase motifs, and showed homology with RuvB DNA helicase of *Thermus thermophilus* and an open reading frame for an unknown 50.5 k protein of *Saccharomyces cerevisiae*. p50 was expressed as a GST-fusion protein and antiserum against the protein was generated. p50 was localized to the nuclear matrix by cell fractionation and immunoblotting. p50 bound to ATP-Sepharose beads. Ultracentrifugation and gel filtration analyses showed that p50 in rat liver and *Xenopus* egg mitotic extracts exists as large complexes corresponding to 697 k and 447 k, respectively. A 50 k protein reactive with p50 antibodies was detected not only in rat liver nuclei, but also in a *Xenopus* egg cytoplasm fraction and a *S. cerevisiae* extract. This suggests that this putative DNA helicase is present in a wide variety of species ranging from yeast to mammals.

Key words: cDNA cloning, DNA helicase, helicase, p50, RuvB.

There is a family of proteins with single *N*-acetylglucosamine residues *O*-linked to serine and threonine residues. These proteins are most abundant in the nucleus (*1*). Important nuclear proteins such as nucleoporin p62 (*2*), RNA polymerase II (*3*), and transcription factors Sp1 (*4*) and HNF1 (*5*) have been shown to be *O*-linked *N*-acetylglucosamine (O-GlcNAc)-bearing proteins. We have isolated O-GlcNAc proteins from a rat liver nuclear matrix fraction by wheat germ agglutinin-Sepharose (WGA-Sepharose) affinity chromatography and characterized it (*6*). This fraction contained many O-GlcNAc-proteins, such as p144 (*7*), p39 (*8*), and a nucleoporin complex comprising p62, p60, and p54 (*6*). Non-glycosylated proteins, such as p92 (*9*) and p50 (*8*), were also isolated from the WGA-Sepharose bound fraction. It was shown that p92 is importin β (*9*). This protein interacts with O-GlcNAc proteins in a nuclear pore complex and acts as a carrier protein in nuclear transport (*9*). On the other hand, partial amino acid sequence analysis of p50 showed that it is an unknown protein.

Then, we cloned the cDNA of p50, analyzed its nucleotide sequence, and deduced the amino acid sequence to elucidate its function. The sequence revealed that the protein is a putative eukaryotic homologue of the bacterial DNA helicase, RuvB. After completion of the sequencing, Kanemaki *et al.* reported the purification of a 49 kDa TBP (TATA-binding protein)-interacting protein and its amino acid sequence (*10*). The sequence was the same as that of our p50. Their report was the first of a putative eukaryotic homologue of bacterial RuvB. RuvB is known to play important roles in the late stages of homologous genetic recombination and the recombinational repair of damaged DNA in prokaryotes (for reviews see Refs. *11-13*), and is an ATP-binding protein that acts as a hexameric complex (*14*).

In this study, we analyzed the molecular shape, subcellular distribution, and ATP-binding activity of the putative DNA helicase, p50, to obtain a molecular basis for analysis of its function. It was shown that the protein occurs as a large complex containing an O-GlcNAc protein and is localized to a nuclear matrix fraction. The protein bound to

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[2] To whom correspondence should be addressed. Phone: +81-25-262-6160, Fax: +81-25-262-6165, E-mail: thori@sc.niigata-u.ac.jp

Abbreviations: bp, base pair; CBB, Coomassie Brilliant Blue; EST, expressed sequence tags; GST, glutathione S-transferase; HRP, horseradish peroxidase; O-GlcNAc, *O*-linked *N*-acetylglucosamine; ORF, open reading frame; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidenedifluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TIP49, 49 kDa TATA-binding protein-interacting protein; WGA, wheat germ agglutinin.

ATP-Sepharose beads. It was also shown that p50 is present in a wide range of eukaryotic species ranging from yeast to mammals.

EXPERIMENTAL PROCEDURES

Buffers and Solvents—Buffer A: 10 mM Tris-HCl buffer, pH 7.4, containing 0.2 mM $MgCl_2$; buffer B: 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM $CaCl_2$, 500 mM NaCl, and 0.2 mM PMSF; buffer C: 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM $CaCl_2$, 1 M NaCl, 1% Triton X-100, and 0.2 mM PMSF; PBS: 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl; and PBS-T: PBS containing 0.05% Tween 20.

Preparation of a Rat Liver Nuclear Matrix Fraction—Rat liver nuclei were isolated from fasting rats as described by Kita *et al.* (*6*). The amount of material derived from 1 A_{260} unit of isolated nuclei (approximately 3×10^6 nuclei) was defined as 1 unit (1 U). The nuclear matrix was prepared from the nuclei as described by Kita *et al.* (*6*). Briefly, isolated nuclei were suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 mM sucrose, 5 mM $MgSO_4$, and proteinase inhibitors: 1 mM PMSF, 2 mM benzamidine, 10 μg/ml of leupeptin, and 5 μg/ml each of antipain, chymostatin and pepstatin A (150-300 U of nuclei/ml). The suspension was treated with DNase I and RNase A (final 125 μg/ml of each enzyme) at 4°C for 2 h and then centrifuged at $800\times g$ for 15 min. A "nuclear matrix fraction" was obtained in the form of a pellet. This fraction contained nuclear membrane proteins and inner insoluble nuclear matrix proteins.

Preparation of a WGA-Bound Fraction—The nuclear matrix fraction suspended in buffer A containing 500 mM NaCl, 4% 2-mercaptoethanol, and proteinase inhibitors was incubated at 4°C for 30 min and then centrifuged at $10,000\times g$ for 15 min. The supernatant was designated the "salt extract fraction" of the nuclear matrix. This fraction (about 8,200 U) was dialyzed against buffer B and then applied to a 5 ml WGA-Sepharose column (Seikagaku, Tokyo) equilibrated with buffer B. The column was washed with buffer B and then buffer C to reduce proteins nonspecifically bound to the resin. Then, proteins bound to the column were eluted with buffer B containing 500 mM GlcNAc. The eluate thus obtained was designated as the "WGA-bound fraction" of the nuclear matrix salt extract.

Purification and Partial Amino Acid Sequencing of p50—p50 was separated from the WGA-bound fraction by reversed phase HPLC in 60% formic acid, followed by SDS-PAGE as described previously (*15*). Proteins on the gel were blotted onto a PVDF filter and then the band corresponding to p50 was subjected to amino acid sequencing of the amino terminal region, the sequence, MKIEEVKST, being obtained. The p50 on another gel was excised and digested with lysylendopeptidase as described previously (*16*). The peptides generated from p50 were purified by C8 reversed-phase HPLC and then the amino acid sequences of the peptides were determined. The following three sequences were obtained: peptide 1, IRAQTEGINISEEALNHLGEIG; peptide 2, VPFCPMVGSEVY; and peptide 3, EHVEEISELFYDAK. A search for these amino acid sequences in protein databases suggested that p50 is a newly discovered protein.

Molecular Cloning of p50—Searches of nucleic acid databases revealed that two partial amino acid sequences (peptides 1 and 3) of p50 match parts of the deduced amino acid sequence of a clone of "rat expressed sequence tags (EST)" (Genbank accession No. H32771). A degenerated oligonucleotide, p50-N: ATGAA(A/G)AT(A/C/T)GA(A/G)GA(A/G)GT(A/C/G/T)(A/G)A(A/G), was synthesized for the amino terminal peptide. Then this and a oligonucleotide of which the sequence was derived from the EST nucleotide sequence, p50-F: AATGCTGTCCTTCCCGTTGA, were used to obtain a cDNA fragment of p50 by PCR amplification of a rat liver cDNA library, and the thus amplified DNA fragment (1.4 kbp) was used to screen a rat liver cDNA library. Through these procedures, we isolated a cDNA clone and sequenced it by the dideoxy chain termination method (*17*). This clone comprised 1,567 bp and contained an open reading frame (ORF) encoding a M_r 50,213 polypeptide comprising 456 amino acid residues (Fig. 1). All the amino acid sequences obtained for the p50 peptides were found in the deduced amino acid sequence of the ORF (underlined region in Fig. 1), and the putative start site sequence matched the N-terminal peptide sequence of p50. From these results, we believe that we have obtained the full-length coding sequence for p50. The sequence contained the helicase superfamily motifs, GXGKT/S (amino acid residues 73-77) and DEXX (amino acid residues 302-305), which are boxed in Fig. 1 (*18, 19*).

Expression and Purification of p50 and a Fragment of It as GST-Fusion Proteins—Two oligonucleotide primers: N, 5′-TATGGATCCTAATGAAGATTGAGGAGGTGAAG-3′ as a 5′ primer, and F, 5′-TTAGAATTCATCCTTCCCGTTGATCTTGGC-3′ as a 3′ primer (F is complementary), were synthesized. Then the sequence between these primers was amplified by PCR with a p50 cDNA clone as a template with pfu polymerase (Stratagene, USA). The thus obtained N/F was digested with *Bam*HI and *Eco*RI, and then inserted into expression vector pGEX-3X (Pharmacia LKB Biotech, Sweden). The expression plasmid, which encodes the p50 fragment fused with glutathione S-transferase (GST), was transfected into *Escherichia coli* BL21 (DE3) cells and then expressed with 0.1 mM isopropyl-thio-β-D-galactoside (Fig. 1). The bacterial cells were collected and disrupted by sonication, which resulted in recovery of the recombinant p50 fragment as inclusion bodies in the precipitate fraction. Then, the inclusion bodies were dissolved in a 8 M urea solution, and renatured by removing the urea by dilution and dialysis as described by Buss *et al.* (*20*). The resulting supernatant was combined with a 1/50 volume of 10% Emulgen 109P (Kao, Tokyo) and then loaded onto a column (1.4×2 cm) packed with 2 ml of glutathione-Sepharose 4B (Pharmacia, Sweden). The column was washed extensively with PBS, and then the bound GST-fused p50 fragment (GST-p50′) was eluted with PBS containing 50 mM glutathione. A GST-fusion protein of the full length p50 (GST-p50) was also expressed and purified the same as for GST-p50′ except that the oligonucleotide primers: N2, 5′-TATGGATCCTAATGAAGATTGAGGAGGTGAAG-3′ as a 5′ primer, and C, 5′-TTAGAATTCAGTCGACATACTTCATGTACTTGTCCTGC-3′ as a 3′ primer, were used.

Preparation of Rabbit Anti-p50 Antiserum—Anti-p50 antiserum was raised in female rabbits by immunization with gel pieces containing the recombinant GST-p50 and Freund's adjuvant (*21*). An IgG fraction prepared from the

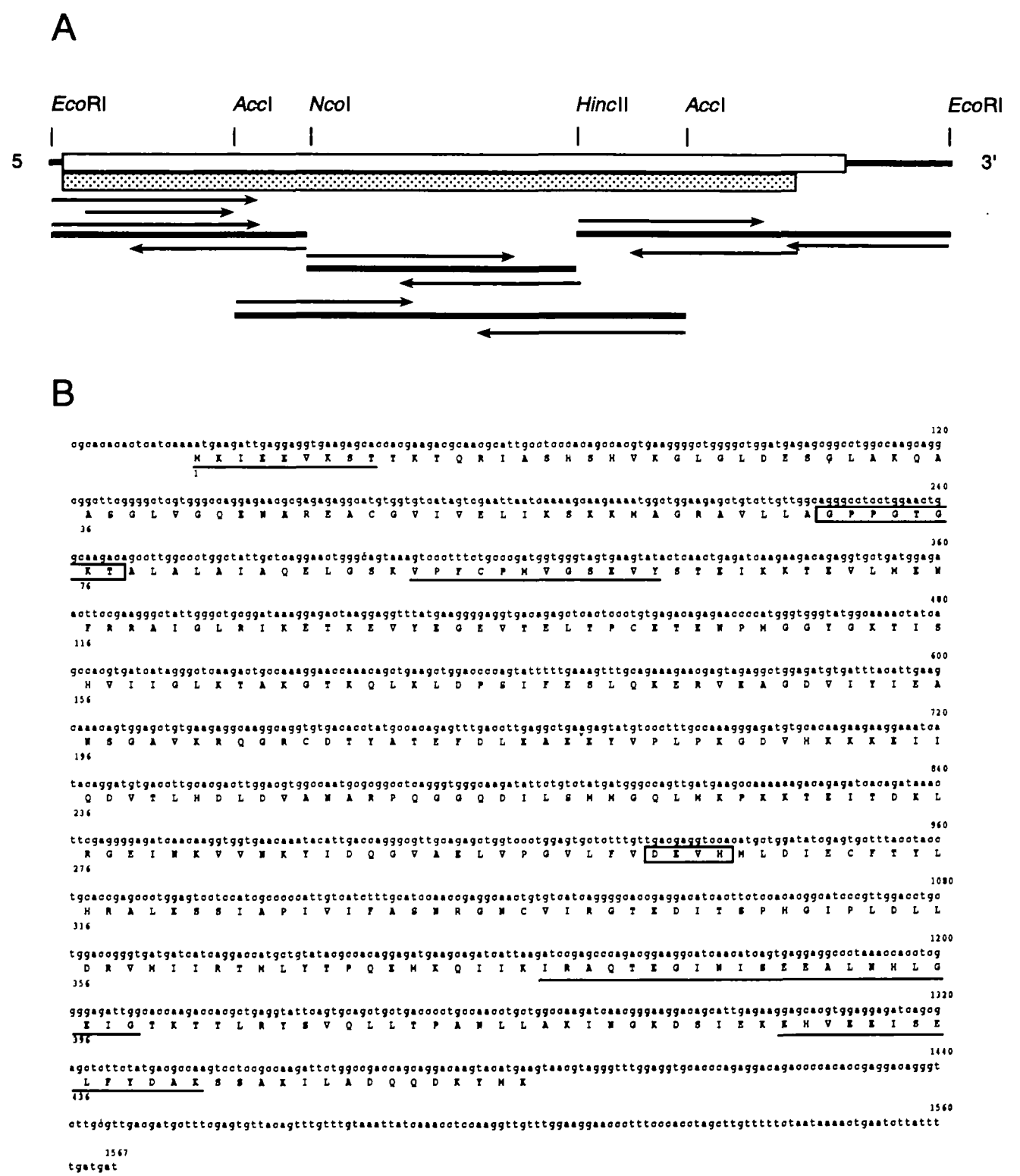

Fig. 1. **Nucleotide and amino acid sequences of p50 cDNA.** (A) Sequencing strategy for rat p50 cDNA. The protein coding region of p50 is represented by an open box. The *Eco*RI/*Nco*I, *Nco*I/*Hinc*II, *Hinc*II/*Eco*RI, and *Acc*I/*Acc*I fragments of the p50 cDNA were subcloned into a Bluescript II SK (−) plasmid vector to sequence the full-length clone. Arrows indicate the sequencing directions and obtained lengths. The hatched box indicates the fragment subcloned into expression vector pGEX-3X for expression of a p50 fragment, GST-p50′. (B) The primary sequence of p50. Nucleotides are numbered on the right above each line. Amino acid residues, represented by a one letter code, are numbered to the left below each line. The sequences obtained for a lysylendopeptidase-digest of p50 are underlined. Helicase motifs are boxed. The GenBank accession number for p50 is AB001581.

antiserum by ammonium sulfate precipitation was treated with GST cross-linked to Sepharose 4B to remove antibodies against the GST moiety of GST-p50. Then the anti-p50 antibodies were purified with GST-p50 cross-linked to Sepharose 4B (*22*).

SDS-PAGE—Samples were separated by SDS-PAGE and visualized by CBB R-250 or silver staining according to the methods of Laemmli (*23*), Blaskesley and Bozei (*24*), and Morrissey (*25*), respectively.

Immunoblotting—Samples separated by SDS-PAGE were electrotransferred onto a PVDF filter. The filter was incubated with PBS containing 5% non-fat dry milk overnight, incubated with the same buffer containing anti-p50 IgG (1 μg/ml) for 1 h, and then washed 3 times with PBS-T for 5 min. Then the filter was incubated with goat anti-rabbit IgG antibodies labeled with horseradish peroxidase (HRP, 1:2,000 v/v) in PBS containing 5% non-fat dry milk for 1 h, washed 5 times for 5 min each with PBS-T, and incubated with the Western blot chemiluminescence reagent, ECL (Amersham, Tokyo), for 1 min. The filter was then exposed to an X-ray film.

Preparation of a Xenopus Egg Extract—An extract of *Xenopus* eggs in the mitotic phase was prepared according to the method of Hirano and Mitchison in the presence of 80 mM sodium β-glycerophosphate and 20 mM EGTA (*26*).

Gel Filtration and Density Gradient Centrifugation—The Stokes' radii of p50 complexes were determined on a Superose 6 HR 10/30 column, 1×30 cm, equilibrated with 50 mM Tris-HCl buffer, pH 7.2, containing 500 mM NaCl, 2.5 mM $MgCl_2$, and 0.2% Emulgen 109P for the WGA-bound fraction, or 50 mM Tris-HCl buffer, pH 7.2, containing 100 mM NaCl, 5 mM $MgCl_2$, 20 mM sodium β-glycerophosphate, 2.5 mM EGTA, and 0.2% Emulgen 109P for the *Xenopus* egg extract. Chromatography was performed at 4°C and 0.4 ml/min. The Stokes' radii of the marker proteins used to calibrate the column were as follows: thyroglobulin, 8.6 nm; catalase, 5.23 nm; bovine serum albumin, 3.62 nm; and ovalbumin, 2.82 nm.

The sedimentation coefficients of the p50 complexes were determined by glycerol density gradient centrifugation. The WGA-bound fractions were loaded onto 4 ml 15-40% glycerol gradients in 50 mM Tris-HCl buffer, pH 7.2, containing 500 mM NaCl, 2.5 mM $MgCl_2$, and 0.2% Emulgen 109P, centrifuged at 110,000×g for 18 h, fractionated, and then analyzed by SDS-PAGE. For analysis of the p50 complexes in the *Xenopus* egg extract, 4 ml 15-40% glycerol gradients in 50 mM Tris-HCl buffer, pH 7.2, containing 100 mM NaCl, 5 mM $MgCl_2$, 20 mM sodium β-glycerophosphate, 2.5 mM EGTA, and 0.2% Emulgen 109P were used. The p50 eluted was determined by immunoblotting. The sedimentation coefficients of the marker proteins used were as follows: thyroglobulin, 20 S; catalase, 11 S; bovine serum albumin, 4.4 S; ovalbumin, 3.7 S; and calbonic anhydrase, 3.0 S.

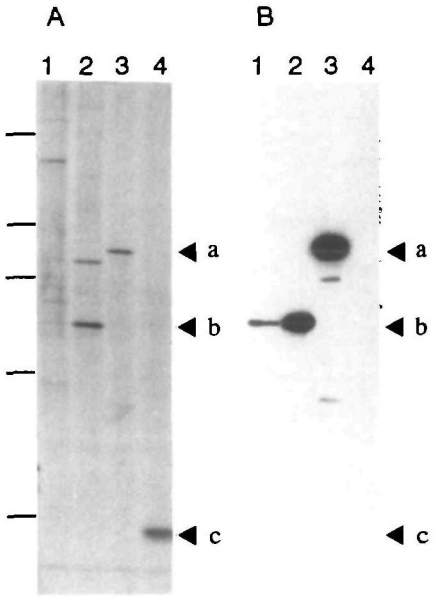

Fig. 2. **Expression of GST-p50′ and preparation of anti-p50 antibodies.** (A) The WGA-bound fraction of a rat liver nuclear matrix salt extract (2 U, lane 1), p50 partially purified by reversed-phase HPLC (5 U, lane 2), GST-p50′ (60 ng, lane 3), and GST (60 ng, lane 4) were separated by 10% SDS-PAGE and then stained with silver. (B) The same samples were separated by SDS-PAGE, transferred to a PVDF filter, and then probed with affinity purified anti-p50 IgG. Arrowheads a, b, and c indicate the positions of GST-p50′, p50, and GST, respectively. The bars at the left indicate the positions of marker proteins of 200 k, 97.4 k, 66.3 k, 43 k, and 28.7 k, from top to bottom.

RESULTS

Subcellular Localization of p50—A p50 fragment (amino acids 1-407) was expressed as a GST-fusion protein (GST-p50′). Antiserum was raised by immunizing rabbits with the recombinant GST-p50′. Then, anti-p50 antibodies were purified by affinity chromatography. The thus purified anti-p50 antibodies reacted specifically with recombinant GST-p50′ and p50 isolated from the rat liver nuclear matrix fraction but not with GST itself (Fig. 2). Then we examined the subcellular localization of p50 using the purified anti-p50 antibodies (Fig. 3). p50 was detected in

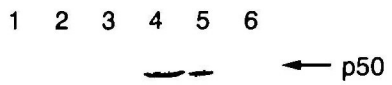

Fig. 3. **Subcellular localization of rat liver p50.** Rat liver subcellular fractions (0.5 U) were separated by 10% SDS-PAGE, transferred to a PVDF filter, and then probed with affinity purified anti-p50 IgG. Lanes: 1, cytosol; 2, mitochondria; 3, microsomes; 4, nuclei; 5, nuclear matrix; and 6, nucleoplasm fraction. The arrow indicates the position of p50.

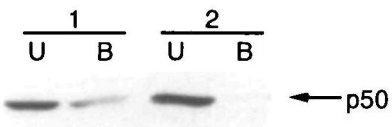

Fig. 4. **Binding of p50 to ATP-Sepharose.** The salt extract of the rat liver nuclear matrix (1.3 U) was incubated with 20 μl of ATP-Sepharose (1) or control Sepharose 4B (2) in 40 mM Tris-HCl, pH 7.4, containing 500 mM NaCl and 1 mM $MgSO_4$ at 4°C for 12 h. Then the bound (B) and unbound (U) fractions were electrophoresed, transferred to a PVDF filter, and probed with affinity purified anti-p50 IgG.

the rat liver nuclear fraction but not in those of other organelles or the cytosol. Of the subnuclear fractions, p50 was localized in the nuclear matrix fraction, which contains proteins that are not solubilized on DNase and RNase treatment (Fig. 3).

ATP Binding of p50—It is known that helicase binds to ATP (27). Therefore, we examined whether p50 could bind to ATP-Sepharose or not. A salt extract of the rat liver nuclear matrix fraction was incubated with ATP-Sepharose, and then the bound and unbound fractions were subjected to SDS-PAGE, followed by immunoblotting with anti-p50 antibodies to detect p50. p50 was detected in the ATP-Sepharose bound fraction but not in the control Sepharose 4B bound fraction (Fig. 4). The band appearing in the lane of the ATP-Sepharose unbound fraction seems to represent p50 overloaded on the resin. These results suggested that p50 is an ATP binding protein.

p50 Homologues in Other Organisms—To examine the presence of p50 homologues in other organisms, immunoblotting with anti-p50 antibodies was performed on extracts of other organisms. An about 50 k protein which is reactive with anti-p50 was detected not only in the rat liver nuclear matrix fraction, but also in a *Xenopus* egg cytoplasm fraction. It was also detected in a whole cell extract of *Saccharomyces cerevisiae*, but not in one of *E. coli* (Fig. 5). These data suggested that p50 homologues are present in a wide range of eukaryotes from yeast to mammals. Then, the databases of *S. cerevisiae* genome DNA sequences were searched and an unknown ORF (YDR109C) encoding a 50.5 k protein was found in chromosome IV (Fig. 6). The rat p50 and yeast ORF were 70% identical in 442 amino acid residues. The amino acid sequence of the yeast ORF also contains conserved helicase motifs, GXGKT/S.....DEXX, which are boxed in Fig. 6. Gene disruption of

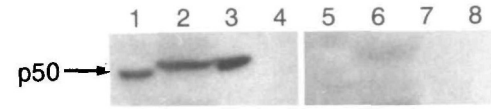

Fig. 5. **Rat p50 homologues in *Xenopus* eggs and yeast.** The *Xenopus* egg cytosol fraction (1, 5), sonicated yeast (2, 6), the rat liver nuclear matrix fraction (3, 7), and sonicated *E. coli* (4, 8) were electrophoresed, transferred to a PVDF filter, and then probed with affinity purified anti-p50 IgG (1-4) or preimmune IgG (5-8). The arrow indicates the position of p50.

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  1'           MKIEEVKSTTKTQRIASHSHVKGLGLDESGLAKQAASGLVGQENAREACGV
                              : :.:.:.:::::::::.::.. .:.::: .::::::::
  1" MVAISEVKENPGVNSSNSGAVTRTAANTHIKGLGLDESGVAKRVEGGPVGQIEAREACGV

 52' IVELIKSKKMAGRAVLLAGPPGTGKTALALAIAQELGSKVPFCPMVGSEVYSTEIKKTEV
     ::.:::.:::.:::.:::: :.:::::::::.:::: :::::::.::::.::.:.::::.
 61" IVDLIKAKKMSGRAILLAGGPSTGKTALALAISQELGPKVPFCPLVGSELYSVEVKKTET

112' LMENFRRAIGLRIKETKEVYEGEVTELTPCETENPMGGYGKTISHVIIGLKTAKGTKQLK
     :::::::::::::::::::::::::::::: ..::::.:::::::::::::.:::.:::::: :.
121" LMENFRRAIGLRIKETKEVYEGEVTELTPEDAENPLGGYGKTISHVIVGLKSAKGTKTLR

172' LDPSIFESLQKERVEAGDVIYIEANSGAVKRQGRCDTYATEFDLEAEEYVPLPKGDVHKK
     :::.:.:::.:.:.:  ::::::::::.::::: :: :.:::::::::.:::::::::.::::
181" LDPTIYESIQREKVSIGDVIYIEANTGAVKRVGRSDAYATEFDLETEEYVPLPKGEVHKK

232' KEIIQDVTLHDLDVANARPQGGQDILSMMGQLMKPKKTEITDKLRGEINKVVNKYIDQGV
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241" KEIVQDVTLHDLDVANARPQGGQDVISMMGQLLKPKKTEITEKLRQEVNKVVAKYIDQGV

292' AELVPGVLFVDEVHMLDIECFTYLHRALESSIAPIVIFASNRGNCVIRGTEDITSPHGIP
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301" AELIPGVLFIDEVNMLDIEIFTYLNKALESNIAPVVVLASNRGMTTVRGTEDVISPHGVP

352' LDLLDRVMIIRTMLYTPQEMKQIIKIRAQTEGINISEEALNHLGEIGTKTTLRYSVQLLT
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361" PDLIDRLLIVRTLPYDKDEIRTIIERRATVERLQVESSALDLLATMGTETSLRYALQLLA

412' PANLLAKINGKDSIEKEHVEEISELFYDAKSSAKILADQQDKYMK
     : ..:::. ...  :  . :.: . :: ::: :.:::
421" PCGILAQTSNRKEIVVNDVNEAKLLFLDAKRSTKILETSANYL
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Fig. 6. **Comparison of the amino acid sequences of rat p50 and the ORF of a yeast 50.5 k protein.** The rat p50 amino acid sequence (upper) shows identity to an unknown ORF encoding a protein of 50.5 k in chromosome IV from *S. cerevisiae* (lower). Colons between the rat and yeast sequences indicate identical amino acid residues and dots indicate conserved residues. Helicase motifs are boxed. The two amino acid sequences are 70% identical in 442 amino acid residues.

TABLE I. **Physicochemical properties of p50 complexes and their subunits.**

	Molecular mass	Sedimentation coefficient (S)	Stokes' radius (nm)	Native molecular mass ($\times 10^{-3}$)	Frictional ratio (f/f_0)	Axial ratio
Rat						
Complex	–	13.9±0.9[a]	11.9±0.4[b]	697±53	2.03±0.06	21.1±1.6
Subunit	50,000[c]	3.3±0.0[c]	3.9±0.0[c]	54±0.3[c]	1.5±0.0[c]	10±0.0[c]
Xenopus						
Complex	–	16.0±0.5[b]	6.63±0.06[b]	447±12	1.31±0.01	5.96±0.30
Subunit	48,000	–	–	–	–	–

[a] $n=4$. [b] $n=3$. [c] These values were reported previously in Ref. 8.

the yeast homologue indicated that the gene encodes an essential product for cell growth (unpublished observation).

Protein Complexes Containing p50—The molecular mass and shape of p50 purified from rat liver in the absence of $MgCl_2$ were studied previously, and the results showed that the purified p50 exists as a monomer (Table I) (*8*). However, when crude p50 in the WGA-bound fraction of the salt extract of the rat liver nuclear matrix was analyzed in the presence of $MgCl_2$ by gel filtration, p50 was eluted in the region of large protein complexes. It is known that the hexameric structure of RuvB is stabilized by Mg^{2+} ions (*28*). Therefore, we determined the sedimentation coefficient and Stokes' radius of the complex in its native form by glycerol density gradient centrifugation and gel filtration in the presence of $MgCl_2$ (Table I). The native molecular mass was calculated from the S-value and the Stokes' radius. The results showed that p50 exists as a large complex (697 k) in the extract. This molecular weight is larger than that of the p50 hexamer (discussed below). The large frictional ratio, 2.03, indicates that the shape of the complex deviates from a globular form. The axial ratio of the complex as prolates, estimated from the frictional ratio using the table of Schachman (*29*), was about 21 (Table I). This value suggested that p50 in the rat liver nuclear extract exists as a large rod-shaped complex. Many nuclear protein complexes are dissociated into their components and solubilized during the mitotic phase of the cell cycle. Therefore, we analyzed p50 complexes in a *Xenopus* egg extract in the mitotic phase. p50 remained as a soluble large complex (447 k) in the mitotic cytosol although it was smaller than that in the interphase extract of rat liver nuclei (Table I).

DISCUSSION

We cloned and sequenced the cDNA for rat liver nuclear protein p50. Its amino acid sequence contained a Walker A ATP/GTP binding motif (*30*): GXGKT/S (amino acids 73-77) (Fig. 1). Helicase motifs (*19, 31*): GXGKT/S..... DEXX (amino acid residues 73-77 and 302-305), were also found in its sequence (Fig. 1). Moreover, the amino acid sequence from the amino terminal to the carboxyl terminal was very similar to that of RuvB DNA helicase of *Thermus thermophilus* (*32*): 21% of the amino acids were identical. The amino acid residues of four portions of p50: 26-85, 298-328, 366-395, and 399-432, were identical with those of RuvB of *T. thermophilus*, *i.e.* 38, 38, 40, and 23%, respectively. It is known that helicase binds to ATP (*27*). Therefore, we examined whether p50 binds to ATP-Sepharose or not, and found that it does bind to ATP-Sepharose (Fig. 4). A homology search was performed in the Swiss Prot database. The high score proteins from the top to the seventh rating comprised RuvB DNA helicases found in many prokaryotes. No RNA helicase appeared within the top twenty. Based on these results, we concluded that p50 is a putative DNA helicase. After completion of the p50 sequencing, a very similar DNA sequence (GenBank accession No. AB002406) was reported by Kanemaki *et al.* (*10*). They cloned a rat 49 kDa TATA-binding protein-interacting protein (TIP49) and sequenced it. The nucleotide sequence of the latter's ORF was completely identical to that of p50, although 5 nucleotides in their noncoding regions are different. Therefore, p50 and TIP49 are identical proteins. They also pointed out the homology of the amino acid sequences of TIP49 and RuvB (*10*). It is well known that DNA helicases play essential roles in DNA replication, repair, and recombination (*11-13*). The well conserved amino acid sequences of p50 that appear in organisms ranging from yeast to mammals (Fig. 6) suggested that p50 helicase is essential for basic functions of eukaryotes. In association with RuvA, RuvB DNA helicase in prokaryotes promotes the branch migration of Holliday junctions during genetic recombination and DNA repair (*31, 33*). Similar functions are expected for the putative p50 DNA helicase. The functions of the putative DNA helicase may be regulated by phosphorylation because consensus sites for phosphorylation by casein kinase II: S/T·X·X·E·X and S/T·X·X·D·X (T^{139}, S^{175}, T^{211}, T^{239}, and T^{267}), and protein kinase C: S/T·X·K/R (T^{9}, T^{12}, S^{58}, T^{74}, T^{163}, T^{272}, S^{331}, T^{402}, and S^{443}), were found in p50.

The subcellular localization of p50 was examined by cell fractionation, immunofluorescence staining, and immunoelectron microscopy. The results with the latter two methods suggested the nuclear localization of p50, although the immunostaining signals were weak (data not shown). On the other hand, experiments involving cell fractionation followed by immunoblotting clearly showed nuclear localization of p50 (Fig. 3, lane 4). This protein was extracted from nuclei with a high salt buffer (Fig. 3, lane 5) but not with a low salt buffer after treatment with DNase and RNase (Fig. 3, lane 6). Therefore, the majority of p50 seems to be bound to a nuclear matrix structure directly or indirectly through an ionic interaction. p50 extracted with 500 mM NaCl remained in a large complex containing a WGA-binding glycoprotein(s) (see below). The binding of p50 and the glycoprotein in the complex is stable in 500 mM NaCl but not in 2 M urea because p50 bound to WGA-Sepharose indirectly was eluted with 2 M urea (*8*). Initially, we expected that p50 is a putative nucleoporin because it was purified from a nucleoporin fraction and complexed with a WGA-binding protein (*8*). However, anti-p50 antibodies did not specifically stain nuclear envelopes, rather they stained the whole nuclei (data not shown). Therefore, we concluded that the majority of p50 is localized in nuclei and binds to a nuclear matrix structure.

p50 exists as a large complex, 697 k, in a rat liver nuclear extract. The complex could bind to WGA-Sepharose, although p50 does not react directly with WGA (*8*). These results suggested that the complex contains a O-GlcNAc glycoprotein(s) as a component(s). It is known that many WGA-binding glycoproteins, such as the Sp1 (*34*), CTF (*34*) and HNF1 (*5*) transcription factors, the largest subunit of RNA polymerase II (*3*), and others act in nuclei (for reviews see Refs. *35* and *36*). Experiments to determine and identify the WGA-binding component of the 697 k complex are now underway. Kanemaki *et al.* showed that TIP49 (p50) binds to the TATA-binding protein (*10*) and exists as a large complex (*37*). Therefore, our 697 k complex may contain the TATA-binding protein. The amount of p50 molecules involved in the complex is not yet known. In the case of bacterial DNA helicases, five classes of helicases, based on sequence homology, are known (*19, 38*). Most 3′→5′ helicases are members of superfamilies I and II. These two families are generally considered to be dimeric. Superfamily III comprises helicases from small DNA and RNA viruses, while superfamilies IV and V

contain 5′→3′ helicases. RuvB helicase, which is a 5′→3′ helicase and a homologue of p50, usually exists as a hexamer (*28*). Therefore, the p50 complex in the rat liver nuclear extract and the *Xenopus* homologue in egg cytosol may both contain 6 mol of p50. However, it is known that the subunit structure changes to a dimer or a dodecamer depending on the protein concentration and the presence of cofactors, such as the RuvA protein, Mg^{2+} ions and ATP (*28*). Therefore, the possibility that the p50 complex contains 2, or 12 mol of p50 can not be excluded. From the molecular masses of the p50 complexes in mitotic and interphase extracts, it was suggested that p50 exists as large complexes in living cells throughout the cell cycle.

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