The human MAT1 protein belongs to the cyclin-dependent kinase-activating kinase complex, which is functionally associated to the transcription/DNA repair factor TFIIH. The N-terminal region of MAT1 consists of a C3HC4 RING finger, which contributes to optimal TFIIH transcriptional activities. We report here the solution structure of the human MAT1 RING finger domain (Met1–Asp65) as determined by 1H NMR spectroscopy. The MAT1 RING finger domain presents the expected βαβ/β topology with two interleaved zinc-binding sites conserved among the RING family. However, the presence of an additional helical segment in the N-terminal part of the domain and a conserved hydrophobic central β strand are the defining features of this new structure and more generally of the MAT1 RING finger subfamily. Comparison of electrostatic surfaces of RING finger structures shows that the RING finger domain of MAT1 presents a remarkable positively charged surface. The functional implications of these MAT1 RING finger features are described.

MAT1 is one of the nine subunits of the human transcription/DNA repair factor TFIIH, which is known to play a crucial role in the transcription of class II genes as well as in DNA repair through the nucleotide excision repair pathway (1). This factor may be resolved in vivo and in vitro into two structural subcomplexes: the TFIIH core and the cyclin-dependent kinase (cdk)1-activating kinase (CAK) complex (2, 3). The CAK complex is composed of the catalytic subunit cdk7, the regulatory subunit cyclin H, and a third partner, MAT1, originally defined as a stabilizing and activating factor (4, 5). This complex is also found in its free form within the cell and preferentially phosphorylates cdk7 known as key components of the cell cycle progression (6). As part of the TFIIH factor, the CAK complex phosphorylates different substrates of the transcription apparatus including TATA box-binding protein, TFIIE, TFIIF, the C-terminal domain of the largest subunit of RNA polymerase II, and regulatory factors such as p53 and some nuclear receptors (7). The cdk7 kinase activity of the CAK complex is stimulated by a combined action of cyclin H and MAT1 binding and cdk7 phosphorylation (8). Moreover, the subunit MAT1 is involved in a substrate selection process choosing either cdk or another transcription apparatus to be phosphorylated (9, 10).

To further investigate the role of the CAK complex in transcription when part of TFIIH and to elucidate the specific role of MAT1, a structural study of all CAK components was undertaken. The crystal structure of cyclin H was solved (11), and a structural model between cdk7 and cyclin H was built (12).

Recently, a combination of sequence analysis and biochemical data showed that MAT1 can be divided into three functional domains: an N-terminal RING finger domain, a central coiled coil domain, and a C-terminal domain rich in hydrophobic residues. Functional analysis revealed that the C terminus strongly interacts in vitro, as well as in vivo, with the cdk7-cyclin H complex and stimulates cdk7 kinase activity (13). The authors showed that the median domain of MAT1 is involved in CAK anchoring to the core TFIIH through interactions with both XPD and XPB helicases. It has also been shown that the deletion of the N terminus, which presents the consensus sequence of a C3HC4 RING finger domain, inhibits the basal transcription as well as the phosphorylation of the C-terminal domain of RNA polymerase II when engaged in a transcription complex (13). This enlightens the potential role of the RING finger domain of MAT1 in the architecture of the preinitiation complex of the transcription.

To complete the functional data available for the MAT1 N-terminal domain and to provide a structural basis for further structure-function relationships, we determined its solution structure using proton NMR spectroscopy. The comparison with previously reported RING finger structures shows that the MAT1 RING finger domain presents a classical βαβ/β topology with a “cross-brace” arrangement of the eight zinc-binding ligands. The MAT1 RING finger domain is characterized by the presence of an additional short α-helix within the N-terminal loop and by an extended basic surface. The functional implications of these features, which are specific to all of the MAT1 RING finger orthologous sequences, are discussed, as are the new insights brought by this fourth high resolution structure of a RING finger.
EXPERIMENTAL PROCEDURES

Expression and Purification of the Recombinant RING Finger Domain—The nucleotide sequence encoding the fragment corresponding to the RING finger domain of MAT1 (Met1–Asp65) was amplified by polymerase chain reaction and inserted into the appropriate Escherichia coli expression vector. The cDNA of the human MAT1 gene was amplified by polymerase chain reaction using a forward primer, which introduces a BamHI site at the 5′ end, and a reverse primer containing a stop codon and an EcoRI site at the 3′ end. After digestion by BamHI and EcoRI (New England Biolabs), the polymerase chain reaction fragment was inserted into the pGEX-4T2 expression vector (Amersham Pharmacia Biotech). A starter culture of 500 ml of LB containing 200 mg/ml ampicillin was inoculated with the E. coli strain BL21(DE3) transformed by the pGEX-4T2 recombinant vector and grown overnight at 37 °C. Cells were pelleted, resuspended in a fresh medium, and used to inoculate 6 liters of LB medium containing 200 μg/ml ampicillin at an $A_{600\, nm}$ of 0.1. Cultures were grown at 37 °C to an $A_{600\, nm}$ of 0.6–0.8, and the expression of recombinant proteins was induced by addition of 0.6 mM isopropyl-1-thio-β-D-galactopyranoside. After 4 h, cells were harvested, washed in buffer A (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 20% glycerol), frozen in liquid nitrogen, and stored at −80 °C.

Cells were resuspended in 100 ml of buffer B (50 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 2.5 mM β-mercaptoethanol and disrupted by sonication for 10 min (pulse 2/8, T = 10 °C) using a 13-mm probe with a VibraCell 72412 sonicator at 30% intensity. The cell extract was then centrifuged for 2 h at 45,000 rpm at 4 °C in a Beckman R60Ti rotor. The soluble extract containing the GST-RING finger recombinant protein was incubated during 1 h at 4 °C with 4 ml of GSH-Sepharose resin (Amersham Pharmacia Biotech) preincubated in buffer B containing 2.5 mM β-mercaptoethanol. The resin was washed in a batch with 40 volumes of buffer B. The adsorbed proteins were eluted with 2 × 4 ml of buffer B containing 30 mM glutathione. The fractions (4 ml) containing the GST-RING finger protein (as judged by SDS-polyacrylamide gel electrophoresis) were pooled, and the GST fusion protein was cleaved with bovine thrombin (Sigma) (3 units per mg of recombinant fusion protein) at 4 °C during 12 h. The sample was concentrated using a Centriprep device with a 3,000-Da cut-off (Amicon). Digestion was stopped by addition of 5 mM Pefabloc (Roche Molecular Biochemicals),

FIG. 1. NMR data of the human MAT1 RING finger domain. A, the number of NOE distances as a function of residue number is shown in violet for intra-residue and in blue, yellow, and red for short, medium, and long range inter-residue, respectively. The presence of intra-residue NOE distances indicates the residues for which stereospecific Hβ assignments were possible. B, stereo view of the $\alpha$βββ fold of the MAT1 RING finger domain with the two zinc ligation sites (ZN1 and ZNII). α-helices and β-strands are displayed with pink boxes and cyan arrows, respectively. Secondary structure NOEs evidencing the three-stranded β-sheet are shown as blue arrows. Hydrogen bond constraints deduced from solvent exchange experiments are indicated by red dashed lines.
and the fraction was then subjected to gel filtration chromatography (Amersham Pharmacia Biotech; 2.6 × 60 cm at a flow rate of 2 ml/min) in buffer C (20 mM Tris-HCl, pH 7.5, 50 mM NaCl). Recombinant RING finger protein-containing fractions were pooled and concentrated on a Centricon prep device with a 3,000-Da cut-off to a final concentration of approximately 2 mg/ml. For NMR studies, the sample was dialyzed against buffer C containing deuterated Tris.

**NMR Spectroscopy**—40 μl of D₂O was added to the 400 μl of the protein solution for the lock, and 2,2-dimethyl-2-silapentane-5-sulfonate was used as the internal chemical shift reference. Homonuclear TOCSY (14), NOE-COSY (16) spectra were recorded at four temperatures (283, 290, 293, and 303 K) on a Bruker DRX600 or DMX750 spectrometers with spectral widths of 7000 Hz (600 MHz) or 8333 Hz (750 MHz) in both dimensions and a relaxation delay of 2 s. Water signal suppression was achieved by presaturation or by using a WATERGATE sequence (17). Slowly exchanging amide protons were identified by recording 70-ms NOESY spectra at 283 K and at different delays after addition of D₂O to the lyophilized sample. Processing was performed on an SGI Octane SE computer using the program FELIX 97 (Biosym Technologies) and on a SGI INDY R5000 computer using XWIN-NMR software (Bruker). Spectra were assigned with the FELIX 97 package (Biosym Technologies) and the XEASY program (18). A single set of resonances was assigned for 65 of the 65 residues of the MAT1 fragment; the two missing residues were the N-terminal Met¹ and Asp². Stereospecific assignments of C²-protons of cysteine residues and the second zinc atom according to tetrahedral bonding patterns. 22

Structure Calculations—A first set of distance constraints was obtained by classifying peak volumes measured on a 70-ms NOESY spectrum recorded at 298 K as strong, medium, and weak, corresponding to distances of 2.7, 3.7, and 5.0 Å, respectively. 60 structures were generated using the restrained simulated annealing protocol implemented in the program X-PLOR 3.851 (20, 21). Eight additional distances of 2.4 Å were added between the two zinc atoms and the S² of cysteine residues and between the side chain of His²⁸ as the fourth ligand for the second zinc-binding site. A second set and Ar²⁴ were then added using the empirical NOE/angle dataset, 616/10; backbone/all atoms rmsd, 0.55/1.02 Å), and promyelocytic leukemia (PML) proto-oncoprotein (Protein Data Bank code 1bor; NOE/angle dataset, 197/28; backbone/all atoms rmsd, 0.68/1.40 Å). Figures showing three-dimensional structures and electrostatic surface representations were prepared with MOLMOL (24).

**RESULTS AND DISCUSSION**

The MAT1 RING Domain Solution Structure—To determine the solution structure of the N-terminal RING finger domain of the human MAT1 subunit, a polypeptide corresponding to residues Met¹–Asp²⁸ was produced. The definition of the domain boundaries was based on mild proteolysis experiments and on the comparison of orthologous sequences. This domain, when expressed as a GST fusion protein in *E. coli*, is soluble and can be easily purified. After removal of the GST tag and subsequent gel filtration, the MAT1 1–65 fragment led to a monodisperse protein solution for the lock, and 2,2-dimethyl-2-silapentane-5-sulfonate was used as the internal chemical shift reference. Homonuclear TOCSY (14), NOE-COSY (15), and DQF-COSY (16) spectra were recorded at four temperatures (283, 290, 293, and 303 K) on a Bruker DRX600 or DMX750 spectrometers with spectral widths of 7000 Hz (600 MHz) or 8333 Hz (750 MHz) in both dimensions and a relaxation delay of 2 s. Water signal suppression was achieved by presaturation or by using a WATERGATE sequence (17). Slowly exchanging amide protons were identified by recording 70-ms NOESY spectra at 283 K and at different delays after addition of D₂O to the lyophilized sample. Processing was performed on an SGI Octane SE computer using the program FELIX 97 (Biosym Technologies) and on a SGI INDY R5000 computer using XWIN-NMR software (Bruker). Spectra were assigned with the FELIX 97 package (Biosym Technologies) and the XEASY program (18). A single set of resonances was assigned for 65 of the 65 residues of the MAT1 fragment; the two missing residues were the N-terminal Met¹ and Asp². Stereospecific assignments of C²-protons of cysteine residues and the second zinc atom according to tetrahedral bonding patterns. 22

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| TABLE I |
| Structural statistics of the MAT1 RING finger |
| **Restraints for calculation** | **Total NOE restraints** | 897 |
| **Intraresidue** | 75 |
| **Sequential (i – j = 1)†** | 286 |
| **Medium range (1 < |j – i| < 4)** | 210 |
| **Long range (|j – i| > 4)** | 326 |
| **Hydrogen bond restraints** | 11 |
| α helix | 7 |
| β-sheet | 4 |
| **Secondary structure statistics** | | |
| rmsd from idealized covalent geometry | | |
| Bonds (Å) | 0.0041 ± 0.0003 |
| Bond angles (°) | 0.78 ± 0.04 |
| Improper torsions (°) | 0.57 ± 0.05 |
| NOE restraints (Å) | 0.0325 ± 0.0035 |
| Dihedral angle restraints (°) | 0.69 ± 0.11 |
| **Final energies (kcal mol⁻¹)†** | | |
| E total | 336.2 ± 43.3 |
| E bonds | 17.2 ± 2.8 |
| E angles | 175.1 ± 18.0 |
| E van der Waals | 50.8 ± 10.9 |
| E NOE | 2.2 ± 0.6 |
| **Ramachandran plot** | | |
| Residues in most favourable regions (%) | 48.1 |
| Residues in additional favourable regions (%) | 30.9 |
| Residues in generously favourable regions (%) | 11.9 |
| Residues in disallowed regions (%) | 3.0 |
| **Coordinate precision** (Å) | | |
| rmsd of backbone atoms (4–62) | 0.667 |
| rmsd of heavy atoms (4–62) | 1.449 |

* Structure statistics refers to an ensemble of 20 structures with the lowest energy from 80 calculated structures.

† rmsd between the ensemble of the 20 structures.
shown in Fig. 1B. The N-terminal fragment of the human MAT1 subunit adopts the ββββ fold typical of RING finger domains and presents an unusual one-turn a helix in its N terminus. The core of the domain consists of a three-stranded antiparallel β-sheet, comprising residues Leu21–Val33 (β1), Thr29–Cys31 (β2), and Arg30–Gln61 (β3) packed along a two-turn a-helix (α2, residues Glu32–Val40).

The triple-stranded β-sheet is clearly defined by an unambiguous pattern of NOEs Ho-HN, Ho-Hα, and HN-HN (Fig. 1C). Slowly exchanging amide protons are observed for residues Met22, Val23, and Gln61 in the β-strands, which indicate that they are hydrogen-bonded. A regular pattern of Ho-HN(i,i+3), Ho-Hβ(i+3), and Ho-HN(i,i+4) NOEs together with upfield-shifted Ho resonances and solvent-protected amide protons define two helical regions (α1 and α2). Some regions of the peptide chain are less well defined (local rmsd, 1 Å) and correspond to loops that link the secondary structure elements, namely loop L1, which encompasses helix...
a1 (Thr12–Arg15), and loop L2 between residues Val40 and Ser56. The Ramachandran plot (data not shown) shows that 97% of the nonglycine and nonproline residues are located in allowed regions; the few residues presenting unusual f and c angles are systematically located in the loop regions.

The RING domain is stabilized by two mononuclear zinc sites separated by 14 Å. Cys6, Cys9, Cys31, and Cys34 form one zinc-binding site (C4), whereas Cys26, Cys46, and Cys49 with His28 form the second zinc-binding site (C3H). The first cysteine pair (Cys6, Cys9) stabilizes the N-terminal part of the peptide. The loop L1 containing the α1 helix is connected to the central β1-strand, which is linked to β2 by a short loop harboring the two zinc ligands Cys26 and His28. A two-turn α-helix (Glu32–Val40) is positioned between the β2-strand and the β3-strand and contains Cys34, which is paired with Cys31 to form the third zinc-ligand pair. Long loop (L2) comprising the fourth pair of zinc ligands (residues Cys46, Cys49) connects the helix α2 to the β3-strand. The overall shape of the MAT1 RING finger domain is found to be slightly elongated, with principal axis lengths of 13.5 × 10.0 × 19.5 Å.

Finally, the MAT1 RING finger core is stabilized by a network of highly conserved hydrophobic residues among MAT1 orthologs (Fig. 2), namely Leu19 in the loop L1; Met22, Leu30, and Val40 in the internal face of the β sheet; Val26, Leu46, and Phe49 in the helix α2; and Leu35 and Phe28 in the loop L2.

Structure Comparison with Known RING Finger Domains—The topology of the three β strands, together with the cross-brace arrangement of the eight zinc-binding residues of MAT1 RING domain, is similar to that observed in two RING atomic resolution structures that have been reported: the structure from the IEEHV protein solved by NMR (30) and the crystal structure of the human recombination-activating protein RAG1 dimerization domain (31). A similar cross-brace arrangement of the zinc-binding residues was also found in the structure of human acute PML proto-oncoprotein (32). The ribbon diagrams of the MAT1 RING structure, together with the three previously reported structures of RING finger domains, are shown in Fig. 3. A data base search for superimposable folds in the Protein Data Bank using the Dali program (33) finds structural similarities between MAT1 and the RING motifs in the RAG1 dimerization domain (31). The best structural homology score is found for the superimposition of the MAT1 structure onto the crystal structure of RAG1. Indeed, both structures can be superimposed for 43 Ca equivalent atoms with an rmsd value of 1.7 Å, whereas the comparison with the solution structure of the IEEHV RING yields 28 equivalent Ca atom positions that superimpose with an rmsd value of 1.99 Å (the structurally equivalent positions are indicated by plain circles in the alignment of Fig. 2B). However, no significant superimposition could be obtained when comparing
the MAT1 RING structure with the solution structure of the RING finger domain from the acute promyelocytic leukemia proto-oncoprotein PML. It is worth mentioning that the weak structural homology observed between the RING finger domains of the human MAT1 protein and PML was also observed when comparing those of RAG1 and PML (31).

The comparison of the MAT1 RING structure with other available RING structures confirms that the consensus C3HC4 zinc-binding sequence defines a conserved structural motif, which constitutes a widely used molecular scaffold. Sequence comparisons of various RING sequences show, however, that this consensus sequence incorporates regions of high sequence diversity with variable spacing between the conserved zinc-binding residues. One of these regions is located between the first two pairs of zinc-binding ligands and encompasses the loop L1. In most RING sequences, this loop contains 10–12 residues, whereas the MAT1 sequence incorporates 16–17 residues (Fig. 2B). The observation of a well-defined secondary structure element (helix $\alpha_1$) in this region is noteworthy and constitutes a specific feature of the MAT1 structure (Fig. 2A). In contrast, the loop L2 containing the fourth zinc ligand pair presents the same conformation as in other RING structures despite the sequence divergence outside the fourth pair of zinc ligands.

The use of stereospecific constraints on most of the $\text{H}^\beta$ methylene protons allows a precise determination of the side chain orientations (angle $\kappa_1$), in particular for the zinc-binding residues. A detailed analysis of the zinc ligation sites in the various RING structures reveals that the second coordination site, ZNII, is well conserved between the different structures. When comparing the ZNII binding sites of MAT1 and RAG1 (Fig. 4), we found a sharp superimposition, with an rmsd of 0.29 Å, of the four MAT1 ZNII ligand side chain heavy atoms (Cys$^{26}$, His$^{28}$, Cys$^{46}$, and Cys$^{48}$) onto the corresponding atoms of RAG1. In the same manner, the ZNII coordination sites of RAG1 and IEEHV can be superimposed with an rmsd of 0.32 Å. The first coordination site is less conserved between the three RING finger structures. Indeed, the superimposition of the Cα of the four ligands (Cys$^{6}$, Cys$^{9}$, Cys$^{31}$, and Cys$^{34}$) onto the equivalent

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**Fig. 5.** Electrostatic surface representations of MAT1, RAG1, IEEHV, and PML structures. Positive and negative charges are shown in blue and red, respectively. The orientations of MAT1, RAG1, IEEHV, and PML RING finger domain surfaces are identical to the one used in Fig. 2. Underlined residues indicate that they are highly conserved within MAT1, RAG1, IEEHV, and PML orthologous sequences.
Ca of RAG1 is poor, yielding an rmsd of 0.57 Å, a value similar to the one obtained when comparing RAG1 and IEEHV (rmsd of 0.54 Å). In the RAG1 structure, the first zinc-binding site is part of a binuclear cluster, with the Cys2⁰⁹ (equivalent to Cys⁸ in MAT1) bridging two zinc atoms. This feature of RAG1 may explain the observed local structure differences around the first zinc-binding site.

Functional Implications of MAT1 Structure—In a recent paper, Busso et al. (13) have established the role of the N-terminal RING finger domain of MAT1 in the activation of transcription in a TFIIFH-dependent manner. They have also observed that the presence of the RING finger domain allows an optimal phosphorylation of the RNA polymerase II C-terminal domain. In agreement with the general role of RING domains in mediating protein-protein interactions (34), it has been suggested that the MAT1 RING domain interacts with other factors within the preinitiation complex of transcription, although no partner has yet been found. When compared with other RING structures, the MAT1 RING domain presents specific features that could be involved in the MAT1 activities.

First, the occurrence of a stretch of conserved hydrophobic residues located in the vicinity of the β1 strand (residues Leu1⁹, Leu2¹, Met2², and Val2³) constitutes an interesting feature. Among these conserved hydrophobic residues, two are involved in the hydrophobic packing of the RING structure, namely Leu¹⁹ and Met²², whereas the two others (Leu²¹ and Val²³) are exposed to the solvent. Such a stretch of hydrophobic residues is unusual in RING sequences (Pfam zf-C3HC4 (35)) and could be related to a regulation of the MAT1 activity.

A second specific structural feature of MAT1 concerns the presence of a structured region including one turn of an α-helix (a1), which corresponds to the sequence insertion between the first and second pairs of zinc-binding ligands, only observed in MAT1 sequences. Interestingly, this helix contains a solvent-exposed tyrosine residue (Tyr¹⁴), which is strictly conserved among all MAT1 sequences, suggesting that the helix α1 might be involved in the packing of the RING domain with another partner. This hypothesis is supported by the comparison of the MAT1 RING structure with the crystal structure of RAG1, which shows that the equivalent part in RAG1 interacts with its N-terminal C2H2 zinc finger (32). Moreover, the solvent-exposed tyrosine could be a potential site for phosphorylation and therefore be involved in activity regulation. Recently, the crystal structure of a complex consisting of a portion of the c-Cbl proto-oncogene protein bound to the ubiquitin-conjugating enzyme UbcH7 and a kinase peptide was reported (36). The structure reveals that the loop L1 of the C-terminal C-chl RING domain interacts closely with both the N-terminal C-chl tyrosine kinase binding domain and the UbcH7 partner, thus emphasizing the functional role of this region in RING domains.

Analysis of the surface electrostatic potential shows that the MAT1 RING domain is highly positively charged because of the presence of several basic side chains of arginines and lysines (Fig. 5). Whereas the extended distribution of the nine positive charges observed at the domain surface is a specific feature of higher eucaryote sequences of MAT1, it is worth noting that the four positively charged residues that are conserved from human to yeast (Lys²⁰, Arg⁴, Lys⁵⁵, and Arg⁵⁹) are located in the same area, forming a positive patch. Two highly conserved acidic residues located on the helix (Glu²² and Asp⁶⁶) form a small negative patch on the exposed side of the helix α2. It is worth noting that the two C-terminal acidic residues (Glu⁶⁴ and Asp⁶⁶) are also conserved but are disordered in the structure. The presence of positively charged patches on protein surfaces seems to be a general feature of the RING finger domains, and the basicity of the MAT1 RING surface is remarkable, as shown in Fig. 5. It must be stressed that most of the mutations that affect the function of PML and IEEHV RING finger domains involve charged residues.

The role of the MAT1 RING finger domain within the transcription complex needs to be studied further by site-directed mutagenesis. Two targets need to be identified from the three-dimensional structure and probed: (i) the positively charged residues that may be involved in the modulation of the CAK phosphorylation activity through electrostatic interactions with either the phosphorylated C-terminal domain, the DNA, or another component of the preinitiation complex, and (ii) the solvent-exposed hydrophobic residues in the strand β1 that may directly affect the stability of the preinitiation complex. It would be of particular interest to know whether these residues are independently related to the two distinct functions of the MAT1 RING finger domain: the phosphorylation of the RNA polymerase II C-terminal domain and transcription activation.

To address these points, the building of specific mutants is currently under way.

Since the first member of the Really Interesting New Gene protein family was identified in 1991 (37), only a few structural data are yet available, partly because of the natural propensity of these domains to aggregate and precipitate when being expressed and concentrated. So far no general structure-activity relationship for the RING finger family has been established.

The solution structure of the N-terminal part of MAT1 is the fourth structure of a RING domain that is now available. These new data provide interesting insights into the structure of the loop region of variable length between the first two pairs of zinc-binding residues that could be useful in other biological contexts. Finally, the structural variability of the loop L1 and the charge distribution at the surface of the RING domains could be essential factors that modulate RING finger activities.

Acknowledgments—We express our gratitude to M. Suzuki and K. Yamasaki at the AIST-NIBHT CREST Center of Structural Biology, Tsukuba, Japan for providing access to the 800-MHz spectrometer and for help during data collection. We are grateful to D. Moras for fruitful discussions, T. Henry for protein expertise, C. Ling for technical assistance, and E. Kellenberger for help with structure calculations. We thank the Institut de Génétique et de Biologie Moléculaire et Cellulaire staff for oligonucleotides and DNA sequencing.

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