Sap1p Binds to Ter1 at the Ribosomal DNA of Schizosaccharomyces pombe and Causes Polar Replication Fork Arrest*

Gregor Krings and Deepak Bastia

From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

Eukaryotic DNA replication forks stall at natural replication fork barriers or termination sites located within the ribosomal DNA (rDNA) intergenic spacer regions during unperturbed DNA replication. The rDNA intergenic spacer of the fission yeast Schizosaccharomyces pombe contains four polar or orientation-specific fork barriers, Ter1–3 and RFP4. Whereas the transcription terminator Reb1p binds Ter2 and Ter3 to arrest replication, the factor(s) responsible for fork arrest at Ter1 and RFP4 remain unknown. Using linker scanning mutagenesis, we have narrowed down minimal Ter1 to 21 bp. Sequence analysis revealed the presence of a consensus binding motif for the essential switch-activating and genome-stabilizing protein Sap1p within this region. Recombinant Sap1p bound Ter1 with high specificity, and endogenous Ter1 binding activity contained Sap1p and comigrated with the Sap1p-Ter1 complex. Circular permutation analysis suggested that Sap1p bends Ter1 and SAS1 upon binding. Targeted mutational analysis revealed that Ter1 mutations, which prevent Sap1p binding in vitro, are defective for replication fork arrest in vivo, whereas mutations that do not affect Sap1p binding remain competent to arrest replication. The results confirm the hypothesis that the chromatin organizer Sap1p binds site-specifically to genomic regions other than SAS1 and support the notion that Sap1p binds the rDNA fork barrier Ter1 to cause polar replication fork arrest at this site but not at SAS1.

Replication forks often stall during normal DNA replication because of encounters with DNA lesions, breaks, or genotoxic stress (1). Stalled forks may be recombinogenic, and regression of these forks without proper restart can lead to genomic instability (1, 2). In addition, forks inevitably encounter natural replication fork barriers or termination (Ter) sites in both prokaryotes and eukaryotes (3–5). In Saccharomyces cerevisiae, polar fork arrest at the rDNA Ter sites presumably coordinates rDNA replication with transcription, regulates rDNA recombination, and controls the formation of extrachromosomal rDNA circles, which have been implicated in aging (6–10). Interestingly, site-specific replication fork arrest within the ribosomal DNA (rDNA) intergenic spacer has been conserved from yeast to humans (11–17). Thus, unraveling the mechanisms and consequences of fork arrest is critical to understanding the physiology of DNA replication and genome stability.

Four fork barriers, Ter1–3 and RFP4, function within the rDNA of S. pombe. Ter1–3 are polar and are located within the intergenic spacer region. Each remains functional when present on a plasmid replicon, suggesting that the sites function as bona fide Ter sites. Furthermore, the intra S-phase checkpoint proteins Swi1p and Swi3p are absolutely necessary for stable fork arrest at Ter1–3. In contrast, RFP4 is located within the terminus of the rDNA transcription unit and is inactive extrachromosomally, suggesting that this barrier may be dependent on rDNA transcription (13).

Most mechanistic knowledge regarding replication fork arrest derives from work in prokaryotic systems in which site-specific binding of terminator proteins to their cognate Ter sites causes polar fork arrest by inhibiting DNA unwinding of replicative helicases and thereby fork progression (18–21). The paradigm that site-specific terminators are required for fork arrest at eukaryotic Ter sites seems to hold true as well, as binding of Fob1p to the rDNA Ter sites is required for polar fork arrest in S. cerevisiae (22, 23). Similarly, mammalian TTF-1 mediates fork arrest at the terminators in mouse rDNA (24). In S. pombe, binding of the transcription terminator protein Reb1p to its repeated cognate binding sites at Ter2 and Ter3 is required to pause replication forks at these barriers (13, 25). It is hypothesized that forks colliding with DNA-bound terminator proteins, such as Reb1p at Ter2 and Ter3, are temporarily stalled at these sites, and that the stalled complex is subsequently stabilized by Swi1p and Swi3p (13). The cause of fork pausing at Ter1 has not been identified. Theoretically, the Ter1 sequence alone could pause replication by folding into a polymerase-stalling secondary structure. Alternatively, Ter1 is likely to bind a terminator protein. Clearly, a necessary prerequisite to studying and understanding the biochemistry of stalled replication forks is to identify all of the factors involved and their respective interactions.

In addition to rDNA fork arrest, natural fork pausing occurs also at two sites near the mating type switch locus mat1. Four transacting factors, rtf1, rtf2, swi1, and swi3 act at the barrier RTS1 to ensure that mat1 is replicated uni-directionally, a prerequisite for imprinting and subsequent mating type switching (26–28). Swi1p and Swi3p likely act at RTS1 to stabilize stalled forks in a manner analogous to their action at the rDNA Ter sites. Forks also pause in a Swi1p- and Swi3p-dependent manner at the site of the imprint (27, 29). Imprinting requires in addition the catalytic subunit of DNA polymerase α Swi7p (30), as well as Sap1p, which binds to its cognate site SAS1, located ~160 bp from the site of the imprint (31, 32). Although necessary for the formation and/or maintenance of the imprint, neither Swi7p nor Sap1p are required for the initial fork pausing event (27, 31). Sap1p is an essential DNA-binding protein and is required for viability independently of its function in
Sap1p-Ter1 Interaction and Replication Fork Arrest

mating type switching (32), perhaps because of its chromatin-organizing function (33). Loss of Sap1p causes defects in chromosome segregation, whereas overexpression of Sap1p is also toxic, causing pleiotropic effects including chromosome fragmentation and abnormal mitosis. These phenotypes occur after the initiation of DNA replication (32). Although these effects are likely initiated by Sap1p binding to numerous sites throughout the genome, the targets remain elusive as no binding sites apart from SAS1 have yet been identified.

In our ongoing efforts to understand more clearly the mechanisms and functional consequences of rDNA replication fork arrest, we have narrowed down the minimal Ter1 site to 21 bp using linker scanning mutagenesis and two-dimensional agarose gel electrophoresis. Analysis of the Ter1 sequence revealed a consensus binding motif for Sap1p. Sap1p bound specifically to Ter1 in vitro, and binding of Sap1p to both Ter1 and SAS1 was shown to bend the DNA. Furthermore, a specific Ter1 binding activity from S. pombe extracts co-migrated with the Sap1p-Ter1 complex and supershift assays suggested that endogenous Sap1p was present within the Ter1-bound complex. Targeted mutagenesis of the Ter1 site revealed that mutations which decreased or inhibited Sap1p binding in vitro also decreased or abolished replication fork arrest in vivo. In contrast, mutations that did not affect Sap1p binding were fully functional for fork arrest. Taken together, these results support the notion that the essential chromatin-organizing protein Sap1p binds within the rDNA at Ter1 and causes polar replication fork arrest at this site.

EXPERIMENTAL PROCEDURES

Strains—S. pombe SP976 (h², ade6-M210, ura4-D18, leu1-32) was used for all yeast experiments. Escherichia coli DH5α was used for all cloning. E. coli BL21 DE3(plysS) was used for expression and purification of His₆-Sap1.

Linker-scanning Mutagenesis—Linker-scanning mutants of the Ter1 region were generated by a PCR-mediated method essentially as described (34), using pIS8F2 as a template. Successive 10-bp sequences of the Ter1-containing fragment in pIS8F2 were thus replaced with the Ncol restriction site. The resulting mutant PCR products were digested with ScaI and SpHI and cloned into plRT2 in blocking orientation with respect to ars1. All primers and oligonucleotides used in this study were purchased from Integrated DNA Technologies. Sequences are available upon request.

Plasmids—Various overlapping fragments were amplified by PCR from Ter1-containing pIS8 (13) and cloned into the unique BamHI site of plRT2. One of these clones, pIS8F2, retained Ter1 activity within a 113-bp fragment as analyzed by two-dimensional agarose electrophoresis (see Fig. 2). To generate pTer1.IRT2, the oligonucleotides GATCTCTC-CATGGATAGCTCTTGCACGCTGTTAACCTCCG and GATCCGGAATTTAACCGACGTGCAAGGACGTATCCATGGGA were annealed, kinased with T4 polynucleotide kinase (New England Biolabs), and cloned into BamHI of pIRT2. All Ter1 mutants were generated in an analogous manner, using the mutant sequences indicated in the text, excepting that SacI/SpHI sites were substituted for the ends to facilitate directional cloning into plRT2. To create pSAS1.IRT2A and pSAS1.IRT2B, oligonucleotides corresponding to SAS1 (38) were annealed, kinased, and ligated into the unique BamHI site of plRT2 in either orientation. pBend2 was a gift from Dr. Dhruba Chattoraj (National Institutes of Health, Bethesda, MD). To generate pTer1.Bend2 and pSAS1.Bend2, oligonucleotides corresponding to the respective sites were annealed, kinased, and ligated into the unique Xbal site of pBend2. To create pHis₆-Sap1, sequences corresponding to the sap1 open reading frame were amplified by PCR and cloned into the BamHI site of pET15b. Plasmids were sequenced by the MUSC Biotechnology Resource Laboratory. Sequences of all oligonucleotides used are available upon request.

Gel Shift and Supershift Assays—To generate probes for gel shift assays, 1 pmol of oligonucleotides corresponding to Ter1, Ter1 mutants, or SAS1 was end-labeled with optikinase (USB) and [γ⁻³²P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences) according to the manufacturer’s instructions. After removal of free γ⁻³²P using G-25 Sephadex spin columns, the oligonucleotides were mixed with a 3-fold excess of cold complementary oligonucleotides in 600 mM NaCl, 1× TE, pH 8, boiled for 5 min, and allowed to return slowly to room temperature overnight. The annealed probes were desalted using G-25 Sephadex spin columns.

For gel shift assays, 3 pmol of labeled double-stranded oligonucleotide probes were incubated with the indicated amounts of His₆-Sap1 or S. pombe crude whole cell extracts in 20 mM Tris, pH 8, 50 mM NaCl, 5% glycerol, and either 100 ng or 1 μg of sheared salmon sperm DNA, respectively, for 5 min at room temperature. Reactions were also performed in 10 mM HEPES, pH 8, 100 mM NaCl, 7 mM β-mercaptoethanol, 0.05% Triton X-100, 5% glycerol with no appreciable difference in DNA binding. Bound and free probes were electrophoresed through 6% native polyacrylamide gels containing 2.5% glycerol using 0.5× Tris borate/EDTA running buffer. Wet gels were directly exposed to x-ray film (Kodak) at −80 °C. For competition experiments, protein was incubated with indicated amounts of excess cold double-stranded oligonucleotides in the same binding buffer prior to addition of the probe.

Supershift assays were performed in a similar manner as gel shift assays, except that bound complexes were further incubated with indicated dilutions of anti-Sap1p antiserum directed at the N terminus of Sap1p (generously provided by Dr. Benoît Angiuli, Institute Pasteur, Paris Cedex, France) for 10 min at room temperature prior to electrophoresis. To test the specificity of the antiserum to Sap1p, the antiserum was depleted of anti-Sap1p antibody by first passing it over His₆-Sap1 immobilized on Talon metal ion affinity beads (BD Biosciences) prior to supershift analysis. Mock-depleted antiserum were prepared in an identical manner by passing it over Talon affinity beads lacking bound His₆-Sap1.

Protein Expression and Purification—His₆-Sap1 was expressed in BL21 DE3(plysS). Briefly, cells were grown to A₆₀₀ nm ~0.6 and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Cells were harvested and lysed on ice with 0.75 mg/ml lysozyme (Sigma) in Buffer A (20 ml 12 mM HEPES, pH 8, 200 mM KCl, 5 mM β-mercaptoethanol, 2 mM MgCl₂, 10% glycerol, containing incomplete (EDTA-free) protease inhibitor mixture (Roche Applied Science)), followed by one cycle of freeze-thawing. Lysed extracts were briefly sonicated with three 10-s pulses and cleared by ultracentrifugation at 30,000 rpm for 30 min at 4 °C. Supernatants were bound in batch for 1 h at 4 °C to 4 ml of Talon metal ion affinity resin beads prewashed in Buffer A containing 20 mM imidazole. The beads were subsequently thoroughly washed with 12 column volumes of the same buffer 5 times for 10 min each plus one wash in Buffer A with KCl adjusted to 400 mM. The beads were then packed into a 10-ml disposable column (Bio-Rad) and again washed with 10 column volumes. Protein was eluted in Buffer A containing 150 mM imidazole (Sigma) into 20 1-ml fractions. Fractions were immediately adjusted to 1 mM dithiothreitol and 5 mM MgCl₂ and analyzed by SDS-PAGE followed by Coomassie Blue staining. Positive fractions were pooled. The protein was >98% pure as judged by Coomassie Blue staining (see Fig. 4A).

Preparation of Crude S. pombe Extracts—Cells were grown to mid-log phase, harvested in ice-cold 133 mM EDTA, 33% glycerol, and frozen...
in liquid nitrogen until use. Thawed pellets were resuspended in an
equal volume of 25 mM Tris, pH 8, 150 mM NaCl, 15 mM EDTA, 5 mM
MgCl₂, 0.1% Nonidet P-40, 10% glycerol, and complete protease inhib-
itator mixture (Roche Applied Science). An equal volume of acid-washed
glass beads (Sigma) was added, and cells were lysed by vigorous vortex-
ing 15 × 1 min each with 1 min pauses on ice inbetween. Glass beads and
cell debris were spun out at 5,000 rpm for 5 min at 4 °C, and the lysate
was subsequently cleared by ultracentrifugation at 40,000 rpm for 1 h at
4 °C. Lysates were either stored on ice for immediate use or frozen in
30% glycerol at −80 °C.

**Two-dimensional Agarose Gel Electrophoresis**—DNA was prepared
and two-dimensional gel experiments were performed as previously
described (13). Radiolabeled probes were directed at the *LEU2* gene of
*pIRT2.*

**DNA Bending**—DNA-bending experiments were performed essen-
tially as described (35). The indicated restriction fragments of pTer1.Bend2 or pSAS1.Bend2 were eluted from agarose gels (Qiagen)
and end-labeled with optikinase (USB) and [γ-32P]ATP (3000 Ci/mmoll) according to the manufacturer’s instructions. Excess free [γ-32P]ATP
was removed by G-25 Sephadex spin columns. Binding was performed
under conditions identical to those used for gel shift assays, and the
complexes were electrophoresed through 10% polyacrylamide gels con-
taining 2.5% glycerol in 0.5× Tris borate/EDTA buffer. Relative mobili-
ies were calculated for the free and bound probes by measuring the
distance each migrated from the wells.

**RESULTS**

**Minimal Ter1 Activity Is Functionally Contained within a 21-bp
Region of the S. pombe rDNA**—We have previously identified four
replication fork barriers, Ter1–3 and RFP4 within the *S. pombe* intergenic
rDNA region (Fig. 1A). Although Ter1–3 were all found to require the
intra-S phase checkpoint proteins Swi1p and Swi3p for activity (13), the
initiating cause for polar fork pausing was only identified for Ter2 and
Ter3, which correspond to binding sites for the RNA polymerase I tran-
scription terminator Reb1p (13, 25, 36). Thus, Reb1p functions also as a
replication terminator at these sites, presumably by pausing the oncom-
ing replication fork as a prerequisite for Swi1p- and Swi3p-dependent
stable fork arrest. However, whether a DNA-binding terminator protein
or DNA structure is responsible for polar fork arrest at Ter1 (Fig. 1B)
remained unanswered. To identify the factor(s) responsible for fork
arrest at Ter1, we chose to first define the Ter1 region at higher resolu-
tion. A 252-bp fragment was previously shown to contain Ter1 activity
when analyzed in a plasmid replicon (pIS8; Ref. 13). To further narrow
down the sequences required, various overlapping fragments were sub-
cloned into the *S. pombe* shuttle vector pIRT2, and replication of the
plasmid was analyzed *in vivo* using two-dimensional agarose electro-
phoresis of PvuII-digested replication intermediates. A 113-bp fragment
(pIS8F2) was found to retain Ter1 activity (Fig. 2B). To define the
site further, this fragment was mutagenized using PCR-based linker
scanning mutagenesis, thereby replacing successive 10 bp of sequence
with NcoI restriction sites. Eight such linker scanning mutants spanning
the majority of IS8F2 are shown schematically in Fig. 2A. Two-dimen-
sional gel analyses identified two mutants (C and D) in which fork arrest
was fully abolished, whereas the other mutants retained fork-arresting
activity (Fig. 2B). Thus, minimal Ter1 seems to be confined to the region
mutated by linker scanners C and D (Figs. 2B and 3A, *arrowheads*). As
expected, when cloned into pIRT2, this region was able to independ-
ently arrest replication (pTER1.IRT2; Fig. 2C). The formal possibility
remains that sequence context flanking this region may contribute to
Ter1 activity, yet these flanking regions are not required for minimal
Ter1 activity.

**Ter1 Contains a Consensus Binding Motif for Sap1p**—Having identi-
fied the minimal sequence required for Ter1 activity, the sequence was
analyzed for potential secondary structures and/or protein binding
sites. Surprisingly, a 10-bp stretch was identified with 100% identity to a
consensus binding motif for the essential mating type switch-activating
and chromatin-organizing protein Sap1p (Fig. 3A; Ref. 37). As depicted
in Fig. 3A, the sequence TTAACGCGAT exactly matches a hypothe-
ized binding site for Sap1p as identified by a stringent synthetic binding
site selection scheme (37). The core TA(A/G)CG motif is found in all
high and low affinity Sap1p-selected binding sites either alone or in
direct or inverted repeat configuration. SAS1, the only other known
binding site for Sap1p, contains three core motifs: two as direct repeats
and one as an inverted repeat. The putative transacting factor acting to
pause replication at this site is depicted by the question mark, *A*, two-dimensional agarose gel electrophoresis of
PvuII-digested replication intermediates of pIS8 containing a 252-bp intergenic spacer
fragment that exhibits polar fork-arresting activity corresponding to Ter1 (left panel,
arrow). Blocking and non-blocking orientations are labeled as B and NB, respectively, and
correspond to the orientation of Ter1 with respect to *ars1* of the plasmid.

**Purified Sap1p Specifically Binds Ter1 in Vitro**—To begin to address
the question whether Sap1p binds to Ter1 and thereby functions as a
terminator protein, the protein was tagged with an N-terminal His₉ tag
and overexpressed and purified to near homogeneity from *E. coli* (Fig.
4A). Bacterial expressed His₉–Sap1p has previously been shown to
retain its conformational integrity and DNA binding activity (38). Gel
shift experiments were performed to assay for Ter1 DNA binding activ-
ity. Indeed, Sap1p bound to Ter1 with relatively higher affinity than it
bound to SAS1, its only previously known genomic binding site (Fig.
4B). Sap1p has been shown to bind to DNA as a dimer. Specifically, an

![Figure 1](image.png)

**FIGURE 1.** Ter1 is a polar replication fork barrier within the *S. pombe* intergenic spacer. A, schematic depiction of the *S. pombe* rDNA, which is arranged as a series of tandem repeated 10.9-kb units on both arms of chromosome III. A detailed view of a single unit is shown. Locations of the fork barriers Ter1–3 and RFP4 are depicted by *numbers 1–4,* respectively. The concave surfaces represent polarity of arrest. *Block arrows* symbolize the RNA transcription units. The directions of RNA transcription and replic-
lication are shown below, as are terminators of transcription and replication. Transcription is terminated near the two Reb1p binding sites. These sites also correspond to the Reb1p-
dependent replication fork barriers Ter2 and Ter3. Note that the polarity of the sites is
reversed for transcription and replication. Ter1 is located upstream of Ter2 and Ter3 with
respect to replication. The putative transacting factor acting to pause replication at this
site is depicted by the question mark, *A*, two-dimensional agarose gel electrophoresis of
PvuII-digested replication intermediates of pIS8 containing a 252-bp intergenic spacer
fragment that exhibits polar fork-arresting activity corresponding to Ter1 (left panel,
arrow). Blocking and non-blocking orientations are labeled as B and NB, respectively, and
correspond to the orientation of Ter1 with respect to *ars1* of the plasmid.
N-terminal dimerization domain has been implicated in inverted repeat binding, whereas a C-terminal dimerization domain is required for SAS1 and direct repeat binding (37). As expected, Sap1p apparently also bound Ter1 as a dimer, as judged by relative migration of the complex in gel shift assays, which was identical to migration of the Sap1p-SAS1 complex (Fig. 4C). Furthermore, deletion of the C-terminal dimerization domain prevented Ter1 binding without affecting inverted repeat binding (data not shown).

The specificity of the Sap1p-Ter1 interaction was studied by competition gel shift experiments. His<sub>6</sub>-Sap1p was incubated with varying amounts of excess competitor wild-type or mutant Ter1 prior to gel shift analysis. For these experiments, a mutant competitor binding site was constructed based on available knowledge regarding Sap1p-SAS1 interaction. Specifically, a double mutation within the core motif (or α-region) has been shown to abolish Sap1p-SAS1 interaction (31). We created an analogous mutation in the Ter1 core motif (DM1), which is similarly defective for binding to recombinant Sap1p (see Fig. 8). Indeed, when His<sub>6</sub>-Sap1p was incubated with excess wild-type non-labeled Ter1, binding to the labeled Ter1 probe was inhibited in a dose-dependent manner (Fig. 4D, left panel). In contrast, the DM1 mutant was unable to compete for His<sub>6</sub>-Sap1p binding, even when present at 60× times molar excess (Fig. 4D, right panel). Therefore, Sap1p bound Ter1 with significant specificity, and analogous bases within the core motif were required for binding to both SAS1 and Ter1.

**Sap1p Bends Ter1 and SAS1**—Several DNA-binding proteins, notably including the *Bacillus subtilis* replication terminator protein RTP, have been found to bend DNA (35, 39). However, in most cases the functional impact of such bending has not been demonstrated. The pBend2 vector, developed by Kim et al. (35) to analyze DNA bending by circular permutation of protein binding sites, was utilized to study Sap1p-mediated DNA bending. The vector contains tandem direct repeats of identical DNA segments containing 17 restriction sites surrounding the cloning site. Restriction by various enzymes thus produces

![FIGURE 2. Linker-scanning mutagenesis of the Ter1 region. A, linker-scanning mutants of the Ter1-containing fragment in pIS8F2 were created by a PCR-based method as described (34). The region was mutated by replacing successive 10-bp stretches of rDNA sequence with the NcoI restriction site. Eight such linker scanners are shown (A–H). NcoI-substituted mutants are symbolized by X. B, PvuII-digested replication intermediates of the parent pIS8F2 plasmid and linker scanning mutants A–H were analyzed by two-dimensional agarose gel electrophoresis. Replication fork barrier activity (arrow) was evident in all mutants except C and D, which were fully defective for fork arrest. C, region encompassing the wild-type rDNA sequence replaced by linker scanning mutants C and D was cloned into pIRT2 in blocking orientation with respect to ara1 and PvuII-digested replication intermediates were analyzed by two-dimensional electrophoresis. As expected, this region was competent to pause the replication fork and thus represents minimal Ter1.](image1)

![FIGURE 3. Ter1 contains a consensus binding site for Sap1p. A, DNA sequence of Ter1. The sequence is oriented to arrest replication forks arriving from the right (arrow) but not the left. Boundaries of the sequence replaced by linker scanning mutants C and D are depicted by arrowheads. The consensus Sap1p binding site TTAACGCAGT is shown in larger font size, and the TAACG core motif is underlined. B, DNA sequence of the conserved intermediate affinity Sap1p consensus binding motif, as previously identified by a binding site selection scheme (37). The TAA/GCCG core motif is underlined and shown in increased font size. Note that the Ter1 sequence reveals 100% identity to the consensus sequence.](image2)

![FIGURE 4. Purified recombinant His<sub>6</sub>-Sap1p binds Ter1 with specificity in vitro. A, 10% SDS-PAGE gel of purified His<sub>6</sub>-Sap1p (*). B, His<sub>6</sub>-Sap1p binds Ter1 in vitro, as detected by gel shift assay. Lanes 1–12 contained 3 fmol of radiolabeled Ter1 probe, as well as 0, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 ng of His<sub>6</sub>-Sap1p, respectively. A distinct gel shift was observed (arrow) even at subequimolar concentrations of Sap1p with respect to Ter1. In comparison, lanes 13–24 contained 3 fmol of radiolabeled SAS1 probe, as well as 0, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 ng of His<sub>6</sub>-Sap1p, respectively. C, electrophoretic migration of the Sap1p-Ter1 complex compared with Sap1p-SAS1. Lane 1, 1.5 fmol of SAS1 plus 10 ng of His<sub>6</sub>-Sap1p; lane 2, 1.5 fmol of Ter1 plus 156 pg of His<sub>6</sub>-Sap1p; D, competition gel shift experiments. His<sub>6</sub>-Sap1p was incubated with increasing amounts of unlabeled wild-type or mutant DM1 Ter1 DNA in addition to radiolabeled Ter1 probe and analyzed by gel shift analysis. All reactions contained 3 fmol of radiolabeled Ter1. Lane 1, Ter1 only; lanes 2–8, Ter1 plus 5 ng of His<sub>6</sub>-Sap1p and 0, 18, 36, 72, 108, 144, 180 fmol of unlabeled wild-type Ter1, respectively; lanes 9–14, Ter1 plus 5 ng of His<sub>6</sub>-Sap1p and 18, 36, 72, 108, 144, 180 fmol of unlabeled mutant DM1 Ter1, respectively.](image3)
fragments with circularly permuted binding sites (35). Ter1 and SAS1 were independently cloned into this vector to produce pTer1.Bend2 and pSAS1.Bend2, respectively. Gel shift assays were performed with His6-Sap1p and various end-labeled restriction fragments containing circularly permuted Sap1p binding sites (Fig. 5A). The relative mobility of the Sap1p-Ter1 complex was inversely proportional to the distance of Ter1 from the ends of the fragment, such that mobility was most retarded when Ter1 was located at the center of the fragment and least affected when Ter1 resided at the ends (Fig. 5, B and C). Similar migration patterns were observed for the Sap1p-Ter1 complex (Fig. 5C). The results are to be expected for a DNA-bending protein and thus clearly reveal that the His6-Sap1p dimer bends Ter1 and SAS1. Interestingly, binding of Ter1 was reproducibly, but not markedly, stronger than SAS1 binding (Fig. 5C).

Characterization of Ter1 Binding Activity from S. pombe Crude Extracts—Because Sap1p bound Ter1 in vitro, we wished to confirm that the endogenous protein also binds Ter1. Crude extracts were prepared from wild-type S. pombe grown to mid-log phase, and the extracts were immediately processed for gel shift assays. The extracts contained a single Ter1 binding activity, which co-migrated with the Sap1p-Ter1 complex (Fig. 6A, compare for example lanes 1 and 3). To study further the specificity of the Ter1 binding activity, gel shifts comparing Sap1p and extract binding to either wild-type or mutant Ter1 probes were performed (see Fig. 8A for locations of the mutations). Importantly, neither Sap1p nor the extract bound either the Δ1 or the DM1 mutants (Fig. 6A, first and second panels). Identical results were obtained with the triple mutant TM1 (data not shown). Furthermore, both Sap1p and extract bound the M7 mutant with greatly decreased affinity as compared with wild-type Ter1 (Fig. 6A, third panel). Thus, the specificity of the Ter1 binding activity from crude S. pombe extracts mirrored the specificity of His6-Sap1p for Ter1 (Fig. 6B).

Last, we performed supershift analysis using aSap1p antisem (33). To ensure that the antibody indeed recognized Sap1p under the experimental conditions used and that this interaction could be identified by supershifting the Sap1p-Ter1 complex, reactions were first performed with recombinant Sap1p. The aSap1p antisem clearly supershifted the Sap1p-Ter1 complex, as evidenced by severely retarded mobility of the complex upon antisem addition (Fig. 6B, arrow). To test the specificity of the supershift, the antisem was first depleted of aSap1p antibody by incubation with recombinant His6-Sap1p immobilized on Talon metal affinity beads. In control reactions, the antisem was passed over the Talon beads lacking bound His6-Sap1p. The depleted and mock-depleted antisem were subsequently used for supershifting experiments. As expected, the aSap1p-depleted antisem did not supershift the Sap1p-Ter1 complex, whereas the mock-depleted antisem did not (Fig. 6B, compare lanes 5 and 6). Identical reactions were subsequently performed with crude S. pombe extracts. The antisem clearly supershifted at least a fraction of the Ter1-bound protein (Fig. 6B, lane 8). As with the recombinant protein, the supershift was specific, as the
αSap1p-depleted antiserum but not the mock-depleted antiserum failed to result in supershifting (Fig. 6B, compare lanes 9 and 10). The results suggest that endogenous Sap1p also binds Ter1.

**Ter1 Mutations That Prevent Sap1p Binding in Vitro Are Defective for Replication Fork Arrest in Vivo**—Although Sap1p is required for mat1 imprinting and mating type switching, a variety of genetic and indirect biochemical evidence suggests that the protein is not involved in fork pausing at SAS1 (27, 40). To confirm these results directly, the SAS1 site was cloned extrachromosomally in both orientations, and replication intermediates were digested with PvuII and analyzed by two-dimensional gels. As expected, no stalled forks were detected in either orientation (Fig. 7).

Having established that recombinant and endogenous Sap1p binds Ter1, we wished to investigate the functional implications of this interaction on replication fork arrest at Ter1. Sap1p is an essential gene (32, 33), thereby preventing analysis of Ter1 activity in sap1-null mutants. Similarly, overexpression of the protein is toxic, apparently causing chromosome fragmentation and rapid cell death (33). In the absence of a suitable selection scheme to select for viable Sap1p mutants, we chose to circumvent these problems by mutating Ter1 and analyzing the targeted mutants for both Sap1p binding in vitro as well as replication fork arrest in vivo, reasoning that, if Sap1p is the terminator protein, then Ter1 mutants defective in Sap1p binding should also be defective for fork arrest. Conversely, mutations not affecting Sap1p binding should remain functional for fork arrest. Nine single point mutants, 1 deletion mutant, 1 double point mutant, and 1 triple mutant spanning the Ter1 region were created for this purpose (Fig. 8A). Mutations were created within the Sap1p core binding motif (M1, M2, and DM1), within the remainder of the conserved Sap1p binding motif (M3, M4), as well as in the region immediately upstream or downstream (M0–M8, Δ1). The triple mutant TM1 combines mutations M1, M2, and M3 within the conserved consensus motif (Fig. 8A). DNA binding was analyzed by gel shift assay. To study replication fork arrest, the mutants were cloned into pIRT2 and analyzed by two-dimensional gel electrophoresis of PvuII-digested replication intermediates. The results are presented in Fig. 8 and in tabulated form in TABLE ONE. Three mutations, Δ1, DM1, and TM1, completely prevented Sap1p binding in vitro. The same mutants were also fully defective for replication fork arrest in vivo (Fig. 8, B and C and TABLE ONE). As TM1 was fully defective in binding even though M1 was only mildly defective in Sap1p binding, and M2 and M3 were fully competent binders (TABLE ONE; see below), these base pairs likely compensate for one another with respect to Sap1p contact. In contrast to the Δ1 deletion mutant, no single point mutation completely abolished Sap1p binding or fork arrest. However, the 3 mutants M1, M5, and M7 were partially defective in Sap1p binding and also partly defective in fork arrest (TABLE ONE). The remainder of the mutants were fully active for both functions (TABLE ONE). Taken together, these results suggest that Sap1p binding in vitro directly correlates with replication fork arrest and are consistent with the notion that Sap1p binding to Ter1 causes replication fork arrest in vivo.
DISCUSSION

The identification of Sap1p as a Ter1-binding protein capable of arresting replication within the S. pombe rDNA raises several interesting points regarding replication fork arrest as well as the potential role of Ter1 in mediating Sap1p-dependent genomic stability functions. It is well established that Sap1p binds to SAS1 located ~160 bp from the imprinting site at mat1 (31) and that the protein is absolutely essential for imprinting yet not involved in fork pausing (Refs. 27 and 40 and this study). Why does Sap1p cause replication forks to pause at Ter1 but not at SAS1? As Ter1 functions extrachromosomally, intrinsic rDNA structure is apparently not implicated. Theoretically, the mode of binding could dictate whether the Sap1p-DNA complex is functional to arrest replication. Sap1p contains two dimerization domains. Domain V, comprising the first 22 amino acids, is required to orient the monomers in a conformation suitable for binding to TAA(A/G)CG inverted repeats, whereas the C-terminal domain IV dictates head-to-tail monomer arrangement and direct repeat recognition (38). However, as neither inverted nor direct core motifs are functional replication fork barriers, this explanation alone is not suitable (data not shown). Another possible protein interaction domains to cause Ter1 fork arrest. As no structurally similar proteins are identifiable by sequence analysis, this possibility is unlikely (41). Instead, it is perhaps most likely that the Sap1p-Ter1 complex assumes a unique arrest-competent conformation relative to other Sap1p-DNA complexes. In this regard, it is interesting to note that Sap1p reproducibly bent Ter1 to a greater extent than SAS1. Although the difference in bending was not drastic, these results suggest that Sap1p may distort Ter1 to a greater extent than SAS1. Whereas the conserved Sap1p motif of both Ter1 and SAS1 is necessary for Sap1p binding (Ref. 31 and this study), perhaps the downstream region, which presumably also contacts the dimer yet differs in sequence between the sites, contributes contacts required to create a competent fork-arresting complex. Notably, a dimer of the B. subtilis terminator protein RTP also binds DNA (39). Experiments to probe the extent of Sap1p-mediated Ter1 distortion and the potential effects on fork arrest are in progress.

Most replication fork barriers are polar, arresting forks from only one direction (reviewed in Ref. 3), and Ter1 is no exception (Ref. 25 and this study). An ongoing question addresses how such polarity is generated. Extensive work in prokaryotic systems suggests that protein-DNA interaction alone is insufficient to generate polarity. Although DNA contact plays a role, terminator proteins apparently also contact the replicative helicase directly to inhibit DNA unwinding (20, 42). In this regard, the asymmetry of the protein-DNA complex becomes crucial for polar fork arrest. Whether similar mechanisms function in eukaryotes remains to be determined. Incidentally, because Sap1p can form at least two types of dimers with differing symmetries, the binding orientation may play a critical role in polarity determination by displaying different protein surfaces to the oncoming replisome. Precedent for such mechanisms is set by the B. subtilis terminator protein RTP (21). The identification of Sap1p as a terminator thus provides an experimental system to study the generation of polarity. Lastly, it will be interesting to identify the replisomal component targeted by yeast terminator proteins such as Sap1p to halt fork progression. The presumptive replicative helicase MCM complex is a likely candidate. Experiments are underway to determine whether Sap1p functions as a polar contrahelicase.

Sap1p is a chromatin-associated protein required for viability independently of its function in mating type switching (32), and is thus the first identified essential terminator protein. Deletion mutants exhibit abnormal mitosis with defective chromosome segregation followed by rapid cell death soon after germination. Similarly, Sap1p overexpression leads to pleiotropic effects including chromosome fragmentation and abnormal mitoses. These effects apparently occur after the initiation of DNA replication (33). As Sap1p is a DNA-binding protein localized within the nucleus (33), it is hypothesized that these effects are initiated through binding of the protein to various sites throughout the genome. However, apart from SAS1, no other Sap1p binding sites have been discovered. Our results suggest that Sap1p may thus maintain genome stability partly through its actions at Ter1. Significantly, the tandem repeats of rDNA provide Sap1p with at least 70–150 Ter1 binding sites. Because of its repetitive nature, the rDNA comprises a relatively large percentage of the genome and segregates independently from the rest of the genomic DNA (43, 44). Thus, maintaining faithful DNA replication and segregation is critical to genome stability. Binding of Sap1p to Ter1 within the rDNA is thus of considerable interest not only with regard to replication fork arrest, but also as it relates to the function of the protein in maintaining chromosome and rDNA stability. In this regard, it is interesting to note that Tof1 and Csm3, the S. cerevisiae homologs of Swi1p and Swi3p, respectively, function in chromosome cohesion. Tof1 and csm3 mutants exhibit cohesion defects manifested through premature chromosome segregation (45). This function is conserved, as C. elegans Tim1 also functions in cohesion (46). As Swi1p and Swi3p are also required for stable fork arrest at Ter1 (13), it will be interesting to determine whether Sap1p affects chromosome stability and segregation through Swi1p and Swi3p at Ter1 and whether fork arrest is required.

Acknowledgments—We thank Dr. Dhruva Chatterjori and Dr. Benito Arcangeli for providing materials.

REFERENCES

1. McGlynn, P., and Lloyd, R. G. (2002) Nat. Rev. Mol. Cell. Biol. 3, 859–870
2. Cox, M. M., Goodman, M. F., Kreuzer, K. N., Sherratt, D. J., Sandler, S. J., and Marians, K. J. (2001) Nature 404, 37–41
3. Bastia, D., and Mohanty, B. K. (1996) in DNA Replication in Eukaryotic Cells (DePamphilis, M., ed) pp. 177–215, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
4. Rothstein, R., Michel, B., and Gangloff, S. (2000) Genes Dev. 14, 1–10
5. Dalgaard, J. Z., and Klar, A. J. (2001) Genes Dev. 15, 2060–2068
6. Kohayashi, T., and Horischu, T. (1996) Genes Cells 1, 465–474
7. Kohayashi, T., Heck, D. J., Nomura, M., and Horischu, T. (1998) Genes Dev. 12, 3821–3830
8. Jehozua, K., and Horischu, T. (2002) Genes Cells 7, 99–113
9. Takeuchi, Y., Horischu, T., and Kohayashi, T. (2003) Genes Dev. 17, 1497–1506
10. Deposser, P. A., Prusty, R., Kaeberlein, M., Lin, S. J., Ferrigino, P., Silver, P. A., Keil, R. L., and Guarente, L. (1999) Mol. Cell 3, 447–455
11. Brewer, B. J., Lockshon, D., and Fangman, W. (1992) Cell 71, 267–271
12. Sanchez, J. A., Kim, S. M., and Huberman, J. A. (1998) Exp. Cell Res. 238, 220–230
13. Kriegs, G., and Bastia, D. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 14085–14090
14. Hernandez, P., Martin-Parras, L., Martinez-Robles, M. L., and Schwartzman, J. B. (1993) EMBO J. 12, 1475–1485
15. Lopez-estrano, C., Schwartzman, J. B., Kramer, D. B., and Hernandez, P. (1998) J. Mol. Biol. 277, 249–256
16. Little, R. D., Platt, T. H., and Schedlakrutz, C. L. (1993) Mol. Cell. Biol. 13, 6600–6613
17. Wiesendanger, B., Lucchini, R., Koller, T., and Sogo, J. M. (1994) Nucleic Acids Res. 22, 5038–5046
18. Sahoo, T., Mohanty, B. K., and Bastia, D. (1995b) J. Biol. Chem. 270, 29138–29144
19. Khatri, G. S., MacAllister, T., Sista, P. R., and Bastia, D. (1989) Cell 59, 667–674
20. Mulugu, S., Potnis, A., Shamsuzzaman, Taylor, J., Alexander, K., and Bastia, D. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 9569–9574
Sap1p-Ter1 Interaction and Replication Fork Arrest

21. Bussiere, D. E., and Bastia, D. (1999) *Mol. Microbiol.* **31**, 1611–1618
22. Mohanty, B. K., and Bastia, D. (2004) *J. Biol. Chem.* **279**, 1932–1941
23. Kobayashi, T. (2003) *Mol. Cell. Biol.* **23**, 9178–9188
24. Gerber, J. K., Gogel, E., Berger, C., Wallisch, M., Muller, F., Grummt, I., and Grummt, F. (1997) *Cell* **90**, 559–567
25. Sanchez-Gorostiaga, A., Lopez-Estrano, C., Krimmer, D. B., Schwartzman, J. B., and Hernandez, P. (2004) *Mol. Cell. Biol.* **24**, 398–406
26. Dalgaard, J. Z., and Klar, A. J. (1999) *Nature* **400**, 181–184
27. Dalgaard, J. Z., and Klar, A. J. (2000) *Cell* **102**, 745–751
28. Dalgaard, J. Z., and Klar, A. J. (2001) *Trends Genet.* **17**, 153–157
29. Vengrova, S., and Dalgaard, J. Z. (2004) *Genes Dev.* **18**, 794–804
30. Singh, J., and Klar, A. J. (1993) *Nature* **361**, 271–273
31. Arcangioli, B., and Klar, A. J. (1991) *EMBO J.* **10**, 3025–3032
32. Arcangioli, B., Copeland, T. D., and Klar, A. J. (1994) *Mol. Cell. Biol.* **14**, 2058–2065
33. de Lahondes, R., Ribes, V., and Arcangioli, B. (2003) *Eukaryot. Cell* **2**, 910–921
34. Gustin, K. E., and Burk, R. D. (1993) *BioTechniques* **14**, 22–24
35. Kim, J., Zwieb, C., Wu, C., and Adhya, S. (1989) *Gene (Amst.)* **85**, 15–23
36. Zhao, A., Guo, A., Liu, Z., and Pape, L. (1997) *Nucleic Acids Res.* **25**, 904–910
37. Ghazvini, M., Ribes, V., and Arcangioli, B. (1995) *Mol. Cell. Biol.* **15**, 4939–4946
38. Arcangioli, B., Ghazvini, M., and Ribes, V. (1994) *Nucleic Acids Res.* **22**, 2930–2937
39. Kralicek, A. V., Wilson, P. K., Ralston, G. B., Wake, R. G., and King, G. F. (1997) *Nucleic Acids Res.* **25**, 590–596
40. Kaykov, A., Holmes, A. M., and Arcangioli, B. (2004) *EMBO J.* **23**, 930–938
41. Bada, M., Walther, D., Arcangioli, B., Doniach, S., and Delarue, M. (2000) *J. Mol. Biol.* **300**, 563–574
42. Manna, A. C., Pai, K. S., Bussiere, D. E., and Bastia, D. (1996) *Cell* **87**, 881–891
43. D’Amours, D., Stegemeier, F., and Amon, A. (2004) *Cell* **117**, 455–469
44. Granot, D., and Snyder, M. (1991) *Cell Motil. Cytoskel.* **20**, 47–54
45. Mayer, M. L., Pot, J., Chang, M., Xu, H., Aneliiunas, V., Kwock, T., Newitt, R., Aebersold, R., Boone, C., Brown, G. W., and Hieter, P. (2004) *Mol. Biol. Cell* **15**, 1736–1745
46. Chan, R. C., Chan, A., Jeon, M., Wu, T. F., Pasqualone, D., Rougvie, A. E., and Meyer, B. J. (2003) *Nature* **423**, 1002–1009