The *Saccharomyces cerevisiae* High Mobility Group Box Protein HMO1 Contains Two Functional DNA Binding Domains*

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High mobility group (HMG) proteins are architectural proteins whose HMG DNA binding domains confer significant preference for distorted DNA, such as 4-way junctions. HMO1 is one of 10 *Saccharomyces cerevisiae* HMG proteins, and it is required for normal growth and plasmid maintenance and for regulating the susceptibility of yeast chromatin to nucleolysis. Using electrophoretic mobility shift assays, we have shown here that HMO1 binds 26-bp duplex DNA with $K_d = 39.6 \pm 5.0 \text{ nM}$ and that its divergent box A domain participates in DNA interactions, albeit with low affinity. HMO1 has only modest preference for DNA with altered conformations, including DNA with nicks, gaps, overhangs, or loops, as well as for 4-way junction structures and supercoiled DNA. HMO1 binds 4-way junctions with half-maximal saturation of $19.6 \pm 2.2 \text{ nM}$, with only a modest increase in affinity in the absence of magnesium ions (half-maximal saturation $6.1 \pm 1.1 \text{ nM}$). Whereas the box A domain contributes modest structure-specific binding, the box B domain is required for high affinity binding. HMO1 bends DNA, as measured by DNA cyclization assays, facilitating cyclization of 136-, 105-, and 87-bp DNA, but not 75-bp DNA, and it has a significantly longer residence time on DNA minicircles compared with linear duplex DNA. The unique DNA binding properties of HMO1 are consistent with global roles in the maintenance of chromatin structure.

High mobility group (HMG) proteins constitute a significant proportion of non-histone proteins of eukaryotic chromatin. They are abundant proteins that are grouped, in part based on their DNA binding characteristics, into three major classes, HMGA, HMG, and HMGN (1–4). HMG proteins contain one or more homologous repeats of the ~80-amino acid sequence HMG box and are classified into two families based on the abundance, function, and DNA specificity of this conserved region (1, 5, 6). The moderately sequence-specific family is typified by transcription factors such as sex-determining factor SRY and lymphoid enhancer factor LEF-1 (7, 8), whereas the non-sequence-specific family is represented by so-called architectural factors HMGB1/2 and the *Saccharomyces cerevisiae* non-histone chromosomal proteins 6A and 6B (9).

The tertiary structures of HMG boxes from sequence-specific and non-sequence-specific proteins have revealed an evolutionarily conserved, common global fold consisting of an L-shaped structure composed of three $\alpha$-helices (10–19). The HMG DNA binding domain, which interacts with ~10 bp of duplex, binds to the minor groove of DNA by partial intercalation of one or two surface-exposed, conserved hydrophobic residues into the base pair stack. Consequently, the DNA is greatly distorted, resulting in a sharp bend and helical underwinding (4, 8, 14, 16, 20). SRY and LEF-1 cause bending by insertion of helix I hydrophobic residues Ile and Met, respectively, into the base pair stack (Fig. 1) (8, 17). HMGB1 contains tandem HMG box domains referred to as box A and box B; DNA-intercalating residues are located at positions 16 and 37 of helices I and II, respectively, and both can contribute to binding affinity (11, 13, 16, 19). HMGB proteins recognize DNA with structural specificity, binding preferentially to distorted DNA such as 4-way junctions, minicircles, and cisplatinated DNA, and they supercoil relaxed, topologically closed DNA in the presence of topoisomerase I (11, 16, 20–25). HMGB proteins have been implicated in diverse biological processes such as transcription, recombination, replication, and DNA repair by virtue of their ability to facilitate assembly of nucleoprotein complexes (1, 3, 5).

*S. cerevisiae* contains 10 HMG box proteins, including the HMGB1/2 homologs NHP6A/B, ~10-kDa proteins with a single HMG box, that have been shown to participate in the RNA polymerase II and III transcription systems (9, 26–28). HMO1 and HMO2 are ~25-kDa HMG box proteins, also identified by homology-based motif prediction programs as having only a single HMG box, in a position corresponding to box B of mammalian HMGB. However, HMO1 also contains an N-terminal box A domain with weak similarity to consensus HMG box domains (Fig. 1). The relative abundance of NHP6A/B and HMO1/2 was recently determined as part of a global analysis of protein expression in yeast. All four proteins are moderately abundant, with an estimated $1.9 \times 10^6$ molecules of HMO1/cell compared with $~4 \times 10^5$ for NHP6A/B and $1.8 \times 10^5$ for HMO2 (29). Strains bearing *HMO1* or *HMO2* mutant alleles are viable, although HMO1 mutant strains have growth defects, compromised plasmid maintenance, and nucleosome-sensitive chromatin, suggesting that HMO1 may play a role in stability of the chromatin structure (30). HMO1 has been shown to interact genetically and physically with FKBP12 prolyl isomerase, a ubiquitous, highly conserved, abundant enzyme that catalyzes a rate-limiting step in protein folding. Dolinski and Heitman (31) found that mutations in *HMO1* and in the *FPR1* gene that encodes FKBP12 are synthetically lethal. A recent study implicates HMO1 as part of the rRNA transcription apparatus, where it was proposed to function...
in a similar capacity as the upstream binding factor, which is an auxiliary factor in the mammalian and amphibian RNA Polymerase I transcription systems. The upstream binding factor has six HMG domains (32). HMO1 has also recently been shown to participate in putative movement control, but its exact role remains to be elucidated (33).

We have shown here that the divergent box A domain does participate in direct DNA contacts and that it contributes modest structure-specific DNA binding to HMO1, whereas box B confers most of the DNA binding affinity. HMO1 displays a longer residence time on constrained DNA minicircles, consistent with its role in the maintenance of chromatin structure.

**EXPERIMENTAL PROCEDURES**

**Cloning and Purification of Proteins**—The gene encoding HMO1 was amplified from yeast genomic DNA using primers modified to introduce Ndel sites at both ends of the PCR product, forward primer, 5'-GGCTGATCATGACACATCG-3' and reverse primer, 5'-AGTACGGCATACTGCGTCG-3' (Ndel sites underlined). The HMO1 gene was cloned into the Ndel site of plasmid pET5a, and plasmid carrying the HMO1 expression was induced by addition of 1 mM isopropyl-

**DNA Binding and Bending Properties of HMO1**

**Alignment of HMG box helices I and II**

| Helix I | Helix II |
|---------|----------|
| HMG1 A box | PRKGMSSA7A~PFVQCTCREEHHK911111 –PSAVNPSRFKKEGGK9211111 |
| HMG1 B box | PKRPESPA5EFGRSCEYPRK14KEGEH –PGLSTGDAVKILGEMWNTNAA |
| NIHGA | PKRALSAYM4FANENRIVSREN –P -DITFCGVCGLKGEKALTF |
| HMGD | PKRPLSAVM4MLNSAREISKREN –P -GKVEVAKRGCGLAWRK0 |
| HMO1 A box | KA6LVSALFLSFLSNaQTAJEE –VDFYNAAECDDEEKBTAFLTE |
| HMO1 B box | PKPKPIVRFAYSAVROELD8Rag1P –PLSTEPTORISKWWKEL |

**LEF-1**

IK KPKINMA3G MLY KEMRANVVAEAC T –LLESAAIANQILRRWHLR |

**SRY**

VKRPMNAG1VRVQRODKRMALAE –P –PNMSKEISQKLYQXMNLTE |

**UBE**

PKKPLTYFPKFEMFEKARYA –KL –P –ENMNLDT1KLKKSKYXELPE |

**Biosciences Storm Phosphorimager** using software supplied by the manufacturer.
and electrophoresed at 2.5 V/cm for 3 h in 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide after electrophoresis.

Electrophoretic Mobility Shift Assays (EMSA) with DNA Minicircles—The 105-bp fragment generated by BspHI digestion of pET5a DNA was 5′-end labeled with T4 polynucleotide kinase and [γ-32P]ATP. DNA minicircles were generated by intramolecular ligation of the 32P-labeled 105-bp DNA fragment with 20 units of T4 DNA ligase in the presence of Thermotoga maritima HU for 2 h at room temperature (34). Samples were treated with Exonuclease III for 1 h at room temperature and the reactions quenched with stop buffer. The deproteinised DNA was purified on a 6% (w/v) native polyacrylamide gel (39:1 acrylamide:bisacrylamide).

EMSA was performed with linear and circular 105-bp DNA. Reactions were incubated at room temperature in 10 μl of reaction buffer containing 5 fmol DNA and varying amounts of HMO1. Where indicated, reactions were performed in the absence of MgCl2. Samples were resolved on prerun 7% (w/v) native polyacrylamide gels (39:1 acrylamide:bisacrylamide) at 4 °C with 0.5× TBE running buffer. Complexes were visualized by phosphorimaging. In DNA competition assays, 10 fmol of 32P-labeled linear or circular 105-bp DNA was incubated with HMO1 for 30 min at room temperature to allow complex formation, followed by addition of varying amounts of linear pUC18 plasmid DNA. Samples were resolved as described above. For calculating fractional complex formation, the region on the gel from the slowest migrating complex to the free DNA was considered as complex.

EMSA with 4-Way DNA Junc- tions—4-Way junctions were constructed using a published protocol (35); the sequence of individual strands was as described (36). The 4-way junction was prepared by annealing the four strands, followed by purification of the junctions on native polyacrylamide gels. To ensure that all oligonucleotides were present, aliquots of the purified junctions were run on denaturing gels. EMSA were performed as described above with 5 fmol DNA and varying amounts of HMO1 or HMO1-BoxA; for experiments with HMO1-BoxA, the reaction buffer was modified to contain only 25 mM NaCl, MgCl2 was omitted, and 0.5% Tween 20 was included. In competition assays, 20- or 1000-fold mass excess of unlabeled linear pUC18 DNA was added after the addition of HMO1. Complexes were resolved and quantified as described above.

EMSA with Linear Duplex DNA and DNA Containing Loops, Nicks, Gaps, or Overhangs—Oligonucleotides were purchased and purified by denaturing gel electrophoresis. Oligonucleotides used to generate constructs with loops, nicks, gaps, or overhangs included a common 37-nt top strand, 5′-CGTAGCCTACGCTTTGGTAGAAATAGTTATGCTTC-3′. To generate 37-bp duplex DNA, complementary oligonucleotides were mixed at equimolar concentrations, heated at 90 °C, and slowly cooled to room temperature. To generate looped DNA, a complementary strand with a pair of 2-nt mismatches with 11-nt spacing (3′-GGATC-GATGCCATGAGAAACATAGTTAATTCGAAG-5′) was annealed to the top strand. A spacing of 11-bp was chosen as it represents the spacing that yielded optimal complex formation with mammalian HMGB1 (37). The 37-bp DNA with a central nick, gap, or a 3′-overhang was prepared as described (36). The 18-bp duplex corresponded to the sequence of the 5′-half of the 37-bp duplex. The sequence of the 26-bp duplex was 5′-CGTGACTACTGTA-AGTCGATGATCCG-3′. EMSA were performed as described above with 5 fmol DNA. The observed fraction of complex formation was corrected for dissociation during electrophoresis, and binding isotherms for HMO1 binding to 26-bp DNA were fitted as described (36). All experiments were done at least in triplicate, and values are reported as the mean ± S.D.

Competition assays involving HMO1-BoxA were performed with 32P-labeled 28-bp DNA. Reactions were incubated at room temperature in 10 μl of reaction buffer containing 10 fmol DNA, 400 fmol B. subtilis HU, or 4 pmol full-length HMO1, and varying amounts of HMO1-BoxA. B. subtilis HU or HMO1 was incubated with DNA at room temperature for 5 min, followed by addition of HMO1-BoxA. Samples were resolved on prerun 7% native gels at 4 °C with 0.5× TBE running buffer. Complexes were visualized by phosphorimaging.

RESULTS

DNA Binding by HMO1 and HMO1-BoxA—Recombinant HMO1 and His6-HMO1 were purified to apparent homogeneity as judged by Coomassie Blue staining of SDS-PAGE gels. For analysis of DNA binding by the divergent box A domain, N-terminal His6-tagged HMO1-BoxA was also prepared (Fig. 2A). CD spectra of both full-length HMO1 and HMO1-BoxA are characterized by negative ellipticities at 208 and 222 nm, indicative of significant α-helical content (Fig. 2, B and C). The qualitatively comparable spectra suggest that HMO1-BoxA is an independently folded domain. The previously reported ability of HMO1 to self-associate (31), evident at higher protein concentrations, was also characteristic of the box A domain for which well resolved CD spectra required the presence of detergent.

DNA binding was analyzed by EMSA. Although no stable complex could be seen on incubation of 18-bp DNA with full-length HMO1 (data not shown), a single complex formed with 26-bp DNA (Fig. 3). No difference was seen between N-terminal His6-tagged and untagged HMO1 (data not shown; all subsequent experiments were performed with untagged HMO1). The apparent dissociation constant $K_d$ for HMO1 binding to 26-bp DNA is 39.5 ± 5.0 nM. HMO1 forms two complexes with 37-bp DNA (Fig. 4A), as seen previously with the mammalian two-HMG box protein HMGB1 (37), suggesting similar site sizes.

FIG. 2. HMO1 and HMO1-BoxA have significant α-helical content. A, SDS-PAGE gels showing purified His$_6$-HMO1 (left) and His$_6$-HMO1-BoxA (right). B and C, CD spectra of HMO1 and HMO1-BoxA, respectively.
Half-maximal saturation is observed at 33.1 ± 3.9 nM HMO1. For reactions performed in the absence of Mg2⁺, the affinity is slightly higher with half-maximal saturation of 15.6 ± 2.3 nM (data not shown). The lower affinity measured in the presence of magnesium ions may not be due to effects caused specifically by the divalent ions but a general effect of raised ionic strength.

Although no complex is detected upon incubation of 26-bp DNA with HMO1-BoxA, a competition experiment in which full-length HMO1 is incubated with 26-bp DNA in the presence of increasing concentrations of HMO1-BoxA shows a reduction in HMO1-DNA complex formation, suggesting that HMO1-BoxA competes for binding to the DNA (data not shown). However, because HMO1-BoxA self-associates, this experiment does not exclude the possibility that HMO1-DNA complex formation is impaired because of interactions between HMO1-BoxA and full-length protein. The competition experiment was therefore also performed using the unrelated architectural protein HU from B. subtilis (Fig. 5A). Consistent with DNA binding by HMO1-BoxA, HU-DNA complex formation is also reduced in the presence of HMO1-BoxA. Efficient competition requires micromolar concentration of HMO1-BoxA, suggesting low affinity binding, consistent with HMO1-BoxA complexes with 26-bp DNA being too unstable to detect following electrophoresis.

**HMO1 and HMO1-BoxA Have Only Limited Preference for Distorted DNA**—HMG proteins have been shown to recognize distorted DNA structures selectively (21, 24, 35, 37, 38). Compared with linear duplex DNA, however, HMO1 did not show binding preference for DNA with loops, nicks, gaps, or overhangs (data not shown); by comparison, the 37-bp looped DNA construct served as a preferred substrate for mammalian HMGB1 (37). Evidently, intrinsic DNA flexibility does not confer a significant energetic advantage for HMO1 to bind DNA. Surprisingly, HMO1 also had only limited preference for 4-way junctions (Fig. 4A). Half-maximal saturation is observed at 19.6 ± 2.2 nM HMO1; for reactions performed in the absence of Mg2⁺, half-maximal saturation is 6.1 ± 1.1 nM. This is in distinct contrast to other HMGB proteins that were shown to bind 4-way junction DNA only in the open square conformation that is preferred in the absence of Mg2⁺ (39). For comparison, B. subtilis HU showed the expected preference for the 4-way junctions compared with linear DNA with a sequence that corresponds to the longest arms of the 4-way junctions, confirming integrity of the 4-way junction construct (Fig. 4B). As shown in Fig. 5B, HMO1-BoxA also exhibits a modest preference for 4-way junction DNA; consistent with self-association of HMO1-BoxA, stable complex formation is enhanced by the presence of detergent. Notably, the formation of detectable complexes with a migration pattern similar to that seen for full-length HMO1 confirms the ability of HMO1-BoxA to engage DNA directly.

When HMO1 was added to reaction mixtures containing both 4-way junction and linear duplex DNA, HMO1 bound with only modest preference to the 4-way junctions (Fig. 6). In competition assays, EMSA was performed with either ³²P-labeled 4-way junctions or ³²P-labeled linear duplex DNA in the presence of unlabeled competitor linear or supercoiled plasmid DNA. HMO1 was competed off the linear and 4-way junction DNA equally efficiently by linear and supercoiled plasmid DNA (data not shown). These assays indicated that HMO1 does not have significant preference for supercoiled compared with linear plasmid DNA. To investigate further the interaction of HMO1 with different DNA topologies, agarose gel retardation assays were performed with negatively supercoiled, relaxed, and linear pUC18 plasmid. As shown in Fig. 7, HMO1 has only modest preference for supercoiled DNA. Regardless of DNA topology, HMO1 causes the entire population of DNA to shift as a rather broad band, suggesting that HMO1 binds without cooperativity. Consistent with its low affinity binding to 26-bp DNA, HMO1-BoxA does not form complexes with plasmid DNA that are detectable following electrophoresis (data not shown).

**HMO1 Exhibits a Longer Residence Time on DNA Minicircles**—DNA minicircles are circularized DNA shorter than the persistence length of ~150 bp. Because the DNA is highly constrained, a diminished need for DNA distortion may lower the free energy of interaction. HMO1 bound 105-bp circular DNA comparably to 105-bp linear DNA (Fig. 8A). However, in DNA competition assays with linear pUC18 plasmid, HMO1 was more efficiently competed off linear 105-bp DNA. At 400-fold excess plasmid DNA, HMO1 still bound 91% of the 105-bp circular DNA as opposed to only 13% of the linear 105-bp DNA (Fig. 8B), suggesting a much longer residence time on the constrained minicircle.

**HMO1 Bends DNA**—Ligase-mediated circularization assays were performed measuring the efficiency with which T4 DNA ligase mediates ring closure of DNA fragments that are shorter than the persistence length (20, 40). The ability of HMO1 to enhance ligase-mediated DNA circularization was qualitatively assessed with 136-, 105-, 87-, 75-, and 65-bp duplex DNA. HMO1 facilitated formation of circles with DNA of 136, 105, and 87 bp, but not 75 or 65 bp (Fig. 9). As expected, initial rates of cyclization were highest for the longer 136-bp DNA; however, the 87-bp DNA produced monomer circles most efficiently. Dimer circle formation and multimerization of the 87-bp DNA occurred in reactions with and without HMO1, although to a lesser extent compared with the other DNA constructs. The 105-bp DNA yielded the least efficient monomer circle formation. However, more than 50% of the DNA formed dimer circles even in the absence of the protein, lowering the effective concentration of monomeric DNA and causing the initial rate and
net yield of monomer circles to be lower than that measured with 87-bp DNA. Secondly, the different cohesive termini of the DNA probes are recognized differentially by DNA ligase. By comparison, *S. cerevisiae* non-histone protein 6A (NHP6A) can form monomer circles with DNA substrates of 66 bp (20), whereas HMGB1/2 and HMG-D can form monomer circles with DNA substrates as short as 59 and 55 bp, respectively, at high protein concentrations (41, 42). The protein concentrations used in our experiments are comparable with the concentrations used for other HMGB proteins; our data therefore indicate that HMO1 bends DNA, although not as effectively as other HMGB proteins.

**HMO1 Does Not Supercoil Relaxed DNA**—Many HMGB proteins insert negative supercoils in the presence of topoisomerase I in topologically closed DNA (25, 30, 43). To assess the ability of HMO1 to supercoole DNA, relaxed closed circular pUC18 DNA was incubated with increasing amounts of HMO1 (Fig. 10). There was no evidence of DNA supercoiling by HMO1, nor did HMO1-BoxA supercoil DNA (not shown). This contrasts with a previous report by Lu *et al.* (30) who, focusing on analysis of HMO1 purified from yeast, reported that HMO1 could indeed supercoil relaxed plasmid DNA.

**DISCUSSION**

Recent studies have shown that the difference between sequence-specific HMGB proteins and the non-sequence-specific homologs depends on individual DNA-intercalating residues and the global features of the HMG box, which determines the mode of DNA recognition. For the tandem HMG boxes of HMGB1, the DNA-intercalating residues are at positions 16 or 37, located in helices I and II, respectively, and both can contribute to the binding affinity of the HMG boxes. Box A has an alanine at position 16, which does not intercalate with the DNA but forms a hydrophobic contact, whereas phenylalanine at position 37 is used as a bending wedge (Fig. 1). Box B has a phenylalanine at position 16 and isoleucine at position 37, both of which are potential intercalating residues. Sequence-specific HMGB proteins have polar residues at position 37 that participate in sequence-specific hydrogen bond formation (8, 16, 42, 44). For both box A and B of HMGB1, their ability to bend DNA is reflected in preferred binding to distorted DNA. Box B, with its 2 DNA-intercalating residues, introduces the greatest bend, whereas box A fails to bend DNA effectively (41, 45). Box A has the greatest preference for distorted DNA due to stacking of the helix II bending wedge on an exposed base pair, whereas the affinity of box B for distorted DNA is only modestly greater than its affinity for duplex DNA (45, 46). Accordingly, the presence of a hydrophobic DNA-intercalating residue in position 16 of helix I appears to be important for bending, whereas a bending wedge in helix II may be required for selective recognition of distorted DNA.

We have found HMO1 to bend DNA but to have only modest preference for distorted DNA structures, including the 4-way junction. The HMO1 box A domain is highly divergent from the consensus, containing a 5-amino acid insertion in the middle of helix III, including a helix-breaking glycine, that is likely to affect its structure and mode of DNA interaction. However, our data showed that the box A domain has significant α-helical content, consistent with an HMG-like fold, and that it is involved in direct DNA contacts (Figs. 2 and 5). DNA bending by HMO1 would be consistent with the presence of hydrophobic residues in position 16 of both HMG boxes and the potential use of Ile-37 of box A as an additional bending wedge. The limited preference of HMO1 for distorted DNA, combined with
the low affinity binding of HMO1-BoxA that is also only modestly enhanced by the presence of pre-bent DNA conformations, suggests that box A contributes the modest structure specificity, whereas box B, which has no hydrophobic residue at position 37, confers most of the DNA binding affinity. The contribution of box A to DNA interactions is also consistent with the occluded site size for HMO1, which fails to form a stable complex on 18-bp DNA but forms two complexes on 37-bp DNA (Fig. 4) as also seen for the mammalian two-HMG box protein HMGB1 (37).

Ligase-mediated circularization of small DNA fragments has been extensively utilized as a means of comparing the DNA bending activities of non-sequence-specific DNA-bending proteins. Both HMGB1/2 and NHP6A/B catalyze formation of 66-bp circles, although NHP6A/B are more efficient (13, 41, 47). This high efficiency has been attributed to the relative stability of NHP6A/B-DNA complexes compared with HMGB1/2 and HU complexes (13, 41) but could also be due to a difference in the bend angle exerted by these proteins, with the angle exerted by NHP6A/B facilitating DNA strand recognition by ligase (13). HMO1 facilitated formation of circles only with DNA longer than 87 bp (Fig. 9), a lower efficiency that may correspond to...
either a reduced bend angle or a short residence time of HMO1 on linear DNA.

Work by Lu et al. (30) reports that HMO1 introduces negative supercoils into relaxed plasmid DNA and that at high concentrations HMO1 inhibits the unwinding reaction. We did not find evidence that HMO1 has the capability to introduce supercoils into relaxed plasmid DNA (Fig. 10). In our work, we used recombinant HMO1, whereas Lu et al. purified HMO1 from yeast on the basis of its association with a DNA helicase activity, potentially resulting in isolation of posttranslationally modified protein (30). SRY, which is a modestly sequence-specific HMGB protein, also failed to supercoil relaxed DNA in the presence of topoisomerase I (45). At position 37 of helix II, where intercalating hydrophobic residues are located for non-sequence-specific HMGB proteins, SRY has asparagine (Fig. 1). Because the HMO1 box B and SRY both lack a hydrophobic sequence-specific HMGB proteins, SRY has asparagine (Fig. 1).

DNA minicircles most likely resemble possible distorted DNA targets, including features of chromatin structure, DNA topology during recombination, or bends introduced during transcription initiation. For HMO1, a much higher mass excess of plasmid DNA was required to compete for binding compared with complexes involving linear DNA (Fig. 8). Presumably, the complex formed with the minicircle is less dynamic compared with complexes involving linear DNA. This increased residence time is probably a consequence of an optimized fit between the minicircle and HMO1. The more stable association of HMO1 with constrained DNA is consistent with its role in maintaining the integrity of chromatin and in assembly of the rRNA preinitiation complex (30, 32).

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