A High Mobility Group Protein Binds to Long CAG Repeat Tracts and Establishes Their Chromatin Organization in Saccharomyces cerevisiae*S

Haeyoung Kim and Dennis M. Livingston1
From the Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455

Long CAG repeat tracts cause human hereditary neurodegenerative diseases and have a propensity to expand during parental passage. Unusual physical properties of CAG repeat tracts are thought to contribute to their instability. We investigated whether their unusual properties alter the organization of CAG repeat tract chromatin. We report that CAG repeat tracts, embedded in yeast chromosomes, have a noncanonical chromatin organization. Digestion of chromatin with the restriction enzyme Fnu4HI reveals hypersensitive sites occurring ~125 bp apart in the repeat tract. To determine whether a non-histone protein establishes this pattern, we performed a yeast one-hybrid screen using CAG repeat tracts embedded in front of two reporter genes. The screen identified the high mobility group box protein Hmo1. Chromatin immunoprecipitation of epitope-tagged Hmo1 selectively precipitates CAG repeat tracts DNAs that range from 26 to 126 repeat units. Moreover, deletion of HMO1 drastically alters the Fnu4HI digestion pattern of CAG repeat chromatin. These results show that Hmo1 binds to CAG repeat tracts in vivo and establish the basis of their novel chromatin organization.

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1To whom correspondence should be addressed: Dept. of Biochemistry, Molecular Biology, and Biophysics, 321 Church St. SE, Minneapolis, MN 55455. Tel.: 612-625-1484; Fax: 612-625-2163; E-mail: livin001@umn.edu

Materials and Methods

Yeast Strains—All yeast strains in this study were derived from strains SSL204 (14) or P69-4 (15). Strain CAG325-126 was generated by one-step gene replacement of trp1-101 in SSL204 with an ARS1-TRP1 fragment in which a CAG repeat tract of 126 repeat units was placed into the NheI site. The repeat tract is oriented so that the strand containing CAG is the lagging strand template when replication begins at ARS1. Derivatives of CAG325-126 with shorter tract lengths were generated by taking advantage of the propensity of yeast to contract long CAG repeat tracts. HMO1 was deleted from strain SSL204 and its derivatives by one-step gene replacement using amplified PCR products of the KanMX4 cassette knock-out of HMO1 (Open Biosystems YSC 1021). Strains CAG101 and SSL520 msh2Δa were previously described (16, 17). Strain Y1H was derived from strain P69-4 (15) by substituting the upstream-activating elements of the GAL promoter regions of ADE2 and lacZ with a CAG repeat tract of 130 repeat units by two-step gene replacement.

Nuclei Isolation and Fnu4HI Digestion—Yeast nuclei were isolated based on the method described by Almer et al. (18). Nuclei prepared by this method were washed in Fnu4HI digestion buffer (20 mM Tris-Ac (pH 7.9), 50 mM KOAc, 10 mM MgAc, 1 mM dithiothreitol), centrifuged, and suspended in Fnu4HI digestion buffer (~5 mg/mL cell). Nuclei in 0.2 ml were digested with 5 or 10 units of Fnu4HI at 37 °C for 10 min. Reactions were stopped by addition of the same volume of stop solution (2% SDS, 50 mM EDTA) followed by incubation with 2 mg/ml proteinase K at 65 °C for 2 h. DNA was purified by phenol/chloroform extraction and ethanol precipitation. DNA was hydrated in 50 μl of TE buffer (10 mM Tris (pH 8.0), 0.1 mM EDTA).

Indirect End Labeling—Purified DNA was digested with EcoRI or EcoRV for the repeat tract embedded at ARS1-TRP1. BanI for the repeat tract located within ADE2, and NheI or Avall for the SPC25 sequence. Fragments were subjected to electrophoresis through a 1% agarose gel and transferred to MagnaGraph nylon membrane (Osmonics) by capillary blotting. DNA fragments were hybridized by standard methods with appropriate probes and detected using a PhosphorImager (Fuji).
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To determine the distance between Fnu4HI digestion sites, we first determined the length of the repeat tract by PCR and electrophoresis of the PCR products through a DNA sequencing gel (supplemental Fig. 1).

Yeast One-hybrid Screen—Strain Y1H was transformed with yeast two-hybrid libraries Y2H-C1, Y2H-C2, and Y2H-C3 (15) using LiAc (19). Transformants were selected on SC-Ade plates followed by a filter-lift assay. Plasmids were isolated from yeast colonies using the yeast DNA preparation kit (Genta Systems) and then transformed into Escherichia coli XL-1 blue electrocompetent cells (Stratagene). The plasmids were purified from bacterial cells and sequenced using the primer pGAD-seq 5'-TTCCATGTGAGGATACC-3'.

Chromatin Immunoprecipitation—HMO1, including 823 bp of sequence upstream of the start codon, was cloned into pRS316 (20). Three FLAG epitopes were appended to the C terminus as described previously (21). The final plasmid was transformed into CAG325-126 hmo1A. ChiP was performed using the methods described by Strahl-Bolsinger et al. (22) as modified by Rick and Bielinsky (23) using the M2 monoclonal antibody (Sigma). Precipitated DNA prepared by this method was hydratated in 50 μl of TE buffer. Prior to the PCR, template DNA was further diluted to 1:10 for the input DNA and 1:2 for immunoprecipitated samples. The PCR primers were as follows: for amplification of the CAG repeat tract, ChIP-CAG 5'-CTTCACTAGCGGAGGCACAGA-3' and ChIP-CTG 5'-AAGGCTAGCGGCAGATCAGGCGC-3'; for amplification of the ADE2 locus, ADE2-3012L 5'-GCATCTCCAGGTTCTTTCCT-3' and ADE2-3469R 5'-GCTTACTGGGACACCCGGACACAG-3'; for amplification of the SPC25 locus, SPC25-1027L 5'-CCAGCGCTGGAGGTATG-3' and SPC25-1511R 5'-GCGCTAAGTGCTTACC-3'; and for amplification of DNA from the diploid containing a repeat tract and a fragment of ADE2 located adjacent to TRP-ARS1, TRP1-1A 5'-AAGGAAATTTCAACAGCGGATCGA-3' and TRP1-1D 5'-TTACGAGGATGAGGATGGAAGAGA-3'. The ADE2 sequence between primers ADE2-3012L and ADE2-3469R was cloned by including an NheI target sequence at the 5'-end of each primer.

RESULTS

Long CAG Repeat Tracts Have a Noncanonical Chromatin Organization—To investigate the chromatin organization of long CAG repeat tracts, we first generated strain CAG325-126 by embedding a CAG tract of 126 repeat units (378 bp) adjacent to ARS1 in Saccharomyces cerevisiae chromosome IV (Fig. 1A). We performed indirect end labeling (IEL) by digesting nuclei with an endonuclease, purifying the DNA, and cutting with a restriction enzyme that flanks the repeat tract. For our analyses we used two probes labeled RI and RV that flank the repeat tract. Analyses we used two probes labeled RI and RV that flank the repeat tract. These sites are 110 bp (±15 bp) apart. The 125-bp separation is distinctly shorter than the 165-bp repeat distance of canonical yeast nucleosomes (26, 27). A shorter tract of 78 repeat units (234 bp) in strain CAG325-78 yields a pattern with hypersensitive sites 110 bp (±10 bp) apart, two near the edges of the repeat tract and one inside the tract (Fig. 1D). The hypersensitive site at the distal end of the repeat tract was confirmed by probing from the opposite direction.

To confirm that this unusual pattern of digestion is not specific to the chromosomal locus, we repeated the analysis with a CAG repeat tract placed at another locus. This was important because the placement of the CAG repeat tract in strain CAG325 is adjacent to ARS1, and this replication origin is known to establish a pattern of phased nucleosomes that might affect the chromatin organization of the adjacent CAG repeat tract (28, 29). Consequently, we performed IEL analysis with chromatin isolated from strain CAG101 in which a CAG repeat tract is
embedded within ADE2 and placed at AR02 in chromosome VII (17). In this strain the CAG repeat tract of 78 repeat units is located greater than 1 kb from a replication origin (30). Digestion of chromatin from this strain with Fnu4HI generates three hypersensitive sites. Two are close to the edges of the repeat tract and the other is ~110 bp (±10 bp) from the first site (Fig. 1E). The CAG repeat chromatin at the aro2::ADE2 locus has an almost identical pattern of Fnu4HI-hypersensitive sites as the pattern generated by the repeat tract of 78 repeat units adjacent to ARS1 (Fig. 1D). These results show that the Fnu4HI digestion pattern is not locus-specific and that CAG repeat tracts are not likely to be condensed by canonical nucleosomes composed of histone octamer cores.

**A Yeast One-hybrid Screen Identifies Hmo1 as a CAG Repeat Tract-binding Protein**—Based on the noncanonical endonuclease digestion pattern, we speculated that proteins other than (or in addition to) core histones bind, either directly or indirectly, to CAG repeat tracts and contribute to the unusual chromatin organization. To identify specific proteins, we performed a yeast one-hybrid screen (31). For this study, we modified a standard yeast two-hybrid strain (labeled Y2H) by substituting a CAG repeat tract of 130 repeat units for the upstream-activating elements of the GAL promoter in front of two reporter genes, ADE2 and lacZ (Fig. 2B) (15). This one-hybrid strain (labeled Y1H) was transformed with yeast genomic libraries Y2H-C1, Y2H-C2, and Y2H-C3 previously prepared for two-hybrid analyses in which yeast genes were fused to the GAL activation domain (AD) (15). Of 2 × 10⁵ transformants, 72 were both Ade2² and positive for β-galactosidase activity. Upon retesting, the clones that gave the strongest signals contained the entire sequence of HMO1 fused to the GAL activation domain by a bridge of 10 amino acid residues. HMO1 encodes a high mobility group box protein in yeast that binds to single and double strand DNA (32, 33).

To confirm that reporter expression results from Hmo1 association with the CAG repeat tract, and not to cryptic promoters near the reporter genes, we transformed the HMO1-AD plasmid into the original yeast two-hybrid strain, containing the GAL promoters in front of the two reporter genes. If reporter expression in Y1H results from the Hmo1-AD fusion protein binding to the CAG repeat tract, neither reporter gene should be expressed in the Y2H strain. Indeed, Y2H transformed with the HMO1-AD plasmid fails to grow, whereas the Y1H strain grows when transformed with the same plasmid (Fig. 2B). The vector containing the GAL AD alone does not support growth of either strain (Fig. 2B). Both Y1H and Y2H transformed with either plasmid grow on leucine-deficient media as expected (Fig. 2C). These results support the conclusion that Hmo1 promotes reporter expression in the Y1H strain by binding directly to the CAG repeat tracts or to proteins that themselves bind directly.

**CAG Repeat Tracts Can Be Precipitated by Antibodies Directed against Epoitope-tagged Hmo1**—To confirm the interaction of Hmo1 with CAG repeat tracts, we carried out ChIP (Fig. 3). We tagged Hmo1 with three FLAG epitopes at the C-terminal end of HMO1 (Hmo1-FL) and expressed the tagged protein in an hmo1 deletion strain (CAG325-126 hmo1Δ). Hmo1-FL is functional because its expression rescues the retarded growth phenotype of the hmo1 deletion strain (data not shown (32)). When we subjected Hmo1-FL-expressing cells that had been treated with formaldehyde to ChIP analysis, a formaldehyde cross-linked chromatin sample from the Hmo1-FL strain (CAG325-126 hmo1Δ [pRS316 HMO1-FL]) or the untagged strain (CAG325-126) were immunoprecipitated with anti-FLAG antibodies (ChIP) or without antibody (Mock). To detect sequences by PCR, template DNA was diluted to 0.025% (1×) or 0.05% (2×) of the starting DNA for input (input) and 2.5% (1×) or 5% (2×) of starting DNA for the precipitated samples (ChIP and mock). Twice as much PCR product of CAG repeat DNA was subjected to agarose gel electrophoresis than PCR products of SPC25 and ADE2. B, formaldehyde cross-linked chromatin and native chromatin were subjected to ChIP analysis. PCR conditions were the same as described for A (see Supplemental Fig. S1). chromatin cross-linked chromatin prepared from an hmo1Δ/hmo1Δ diploid. One copy of chromosome V carries a repeat tract of 55 repeat units and one carries a 497-bp portion of ADE2 (see A and B) at the same Yel site adjacent to TRP1 ARS1. Primers originate in the TRP1 ARS1 sequences common to both chromosomes. D, formaldehyde cross-linked chromatin prepared from a strain that had both a CAG repeat tract adjacent to TRP1-ARS1 and a repeat tract embedded in ADE2 were subjected to ChIP.

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treated with the cross-linking agent formaldehyde (Fig. 3B). This non-cross-linked chromatin was prepared in a solution with a moderate salt concentration (140 mM NaCl) and a high detergent concentration (1% Triton X-100) that should disrupt weak interactions. The ability to immunoprecipitate the CAG repeat tract without the need to cross-link the protein to the DNA indicates the high affinity that Hmo1 has for the CAG repeat tract.

To investigate further the affinity of the association, we immunoprecipitated Hmo1-FL from a hmo1Δ/hmo1Δ diploid strain in which one copy of chromosome IV bears a CAG repeat tract of 55 repeat units within the NheI site adjacent to ARS1, and the other copy of chromosome IV harbors a 497-bp segment from ADE2 cloned into the same NheI site. A larger percentage of the chromosomal DNA containing the repeat tract is precipitated than the chromosomal DNA containing the ADE2 fragment (Fig. 3C).

Another indication of the affinity of Hmo1 is the ability to precipitate repeat tracts at different chromosomal locations and to short repeat tracts. The one-hybrid studies show that it binds either directly or indirectly to long repeat tracts embedded in the tracts. The one-hybrid studies show that it binds either directly or indirectly to long repeat tracts at different chromosomal locations and to short repeat units (Fig. 4A). All three repeat tracts precipitate, showing that Hmo1 binds to tracts that comprise as few as 78 bp, shorter than the length of DNA wrapped around a histone octamer core. Thus, Hmo1 likely binds to tracts of different lengths and locations.

Deletion of HMO1 Alters the Chromatin Structure of CAG Repeat Tracts—Both the yeast one-hybrid screen and the ChIP analysis provide clear evidence that Hmo1 interacts with CAG repeat tracts. To determine whether Hmo1 is responsible for the unusual chromatin organization of long CAG repeat tracts that we detected by Fnu4HI digestion, we performed IEL on the CAG repeat chromatin from the derivative of CAG325-126 in which HMO1 had been deleted (Fig. 5A). The pattern of Fnu4HI-hypersensitive sites in the CAG repeat tract of the chromatin from the hmo1 deletion strain is strikingly different from that of the HMO1 strain (Fig. 5, A and B). Instead of two strong hypersensitive sites within the interior of the repeat tract, only one weak site appears. Furthermore, this interior site in the hmo1Δ chromatin is not positioned at the location of either of the two sites in the HMO1 chromatin. This is best seen when the downstream R1 probe is used for IEL (Fig. 1A and Fig. 5A). The distance between the interior site and the sites at the borders is ~165 bp (±15 bp), which is consistent with the repeat distance of canonical yeast nucleosomes. Whether this altered pattern results from canonical histone octamer cores will require further examination. Whatever the chromatin state in the absence of Hmo1, the change in the Fnu4HI digestion pattern caused by its absence strongly supports its presence in CAG repeat chromatin.

To substantiate the specificity of the disruption of the Fnu4HI digestion pattern by deletion of HMO1, we carried out two additional control studies. First, we examined whether deletion of another potential CAG repeat binding protein would produce the same disruption. Human MSH2 has been shown to bind in vitro to a substrate generated by denaturing a CAG repeat tract and permitting it to realign (34). Consequently, we investigated CAG repeat chromatin in a derivative of CAG325-126 in which MSH2 had been deleted. CAG repeat tract chromatin isolated from the msh2 deletion strain was digested with Fnu4HI and analyzed using IEL analysis (Fig. 5C). Deletion of MSH2 does not alter the pattern of Fnu4HI-hypersensitive sites in CAG repeat chroma-
A potential model showing that Hmo1 may bind adjacent to a tetramer of core histones H3 and H4. Our working model shows that H2A and H2B are displaced from CAG repeat tract chromatin by Hmo1. This is consistent with the evidence that the yeast ribosomal DNA promoter appears devoid of H2A and H2B (42) and that the necessity of Hmo1 for promotion (43) suggests its binding to the abbreviated cores.

The formal possibility exits that Hmo1 does not bind directly to the DNA but is tightly bound to histones or other tightly bound proteins that are bound to the CAG repeat tract. This possibility seems remote for a number of reasons. First, Hmo1 has two DNA binding domains and binds to both single strand and double strand DNA (33). Second, Hmo1 has never been found to interact with the core histones. Third, we precipitated the CAG repeat tract using antibody directed against the epitope attached to Hmo1 without benefit of formaldehyde treatment. Fourth, the observation that the CAG repeat chromatin becomes more refractory to Fnu4HI digestion when HMO1 is deleted means that either it binds directly or drastically alters the configuration of the proteins to which it binds (Fig. 5, A and B). Although the question of direct binding is important, it does not diminish the conclusion that Hmo1 strongly associates with CAG repeat chromatin and establishes its sensitivity to Fnu4HI digestion.

The widespread presence of HMG box proteins in nature means that our results should have broader implications. Hmo1 has both a B box and an A box and is similar to HMGBI/2 in humans (32, 33). Some HMG proteins exhibit sequence or structure specificity including HMGB1 and -2 that bind to poly(CA) in a four-stranded complex and (GGA/TCC)11 in a triple-stranded complex (35, 36). More notably, the HMG box protein SRY binds tightly to a CA repeat tract and weakly to a CAG repeat tract (37). Thus, HMG box proteins might organize CAG repeat tract chromatin in humans.

Although studies using purified core histones have shown that they preferentially reconstitute on CAG repeat tract DNA (8–12), some studies indicate that the binding may be anomalous. In particular, the studies by Godde and Wolfe (9) show that only six repeat units are needed to effect binding and that the affinity of binding does not increase with repeat length, at least for repeat tracts that can accommodate a single octamer core. Our studies have been done in vivo, where core histones must compete with other DNA-binding proteins. Whether Hmo1 substitutes for core histones or augments their binding in the formation CAG repeat chromatins is uncertain. HMG proteins are thought either to bind adjacent to core histone octamers or to the DNA wrapped around the cores (38, 39). When HMG proteins bind adjacent to histone octamer cores, they increase the amount of DNA protected from nuclease digestion (40, 41). In the case of CAG repeat tract chromatin, the length of DNA protected in the chromatin is shorter than that protected by normal nucleosomes, suggesting that they are unlikely to serve as linkers to octamer cores. We look to the chromatin organization of the ribosomal DNA promoter in yeast as a possible model for the organization of the CAG repeat chromatins. This promoter has H3 and H4 bound to it but appears devoid of H2A and H2B (42). Further work has shown that Hmo1 is needed for RNA polymerase I transcription of ribosomal DNA suggesting that Hmo1 may have replaced H2A and H2B (43). Thus, we present a model in which Hmo1 replaces histones H2A and H2B in the complex of proteins that condenses the CAG repeat tract (Fig. 7). Possibly, Hmo1 serves as a linker between tetramer cores of histones H3 and H4 that have been shown in reconstitution studies to provide protection of 120 bp of DNA (44).
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Based on the correlation in the difference in affinity of core histones for pure and interrupted repeat tracts with the difference in their respective genetic stability, the postulate has been made that chromatin organization contributes to CAG repeat tract instability (8). We tested whether the alteration in the chromatin organization that we observed in the hmo1Δ strain affects repeat tract stability in our tester strain (17). We did not find greater instability in the hmo1Δ strain than in our wild-type strain.9 The negative result probably reflects the fact that chromatin proteins are displaced from the DNA as the replication fork passes and that the excess DNA synthesis needed to expand repeat tracts is the fault of enzymes involved in the dynamic processes of replication, particularly those that are involved in Okazaki fragment joining (45–49). By having shown that CAG repeat tract chromatin is based on the binding of an HMG box protein, we will be curious to learn whether all, or a subset, core histones bind with Hmo1 on long CAG repeat tracts.

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