THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 33, Issue of August 13, pp. 23027–23034, 1999
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Printed in U.S.A.

Histone Acetyltransferase HBO1 Interacts with the ORC1 Subunit of the Human Initiator Protein*

(Received for publication, April 12, 1999, and in revised form, May 12, 1999)

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The origin recognition complex (ORC) is an initiator protein for DNA replication, but also effects transcriptional silencing in *Saccharomyces cerevisiae* and heterochromatin function in *Drosophila.* It is not known, however, whether any of these functions of ORC is conserved in mammals. We report the identification of a novel protein, HBO1 (histone acetyltransferase binding to ORC), that interacts with human ORC1 protein, the largest subunit of ORC. HBO1 exists as part of a multisubunit complex that possesses histone H3 and H4 acetyltransferase activities. A fraction of the relatively abundant HBO1 protein associates with ORC1 in human cell extracts. HBO1 is a member of the MYST domain family that includes *S. cerevisiae* Sas2p, a protein involved in control of transcriptional silencing that also has been genetically linked to ORC function. Thus the interaction between ORC and a MYST domain acetyltransferase is widely conserved. We suggest roles for ORC-mediated acetylation of chromatin in control of both DNA replication and gene expression.

The origin recognition complex (ORC)[1] is a key protein for the initiation of DNA replication in eukaryotes. Initially identified in *Saccharomyces cerevisiae* (1), ORC is a multisubunit protein composed of six polypeptides that binds to yeast replication origins *in vivo* and *in vitro* and is essential for the initiation of DNA replication (2). Homologues of *S. cerevisiae* ORC subunits have been identified in *Schizosaccharomyces pombe, Drosophila melanogaster*, *Arabidopsis thaliana, Xenopus laevis*, and human cells (3–14). *Xenopus* ORC is necessary for chromosome replication in egg extracts (7, 8, 15–18). Moreover, *Drosophila* ORC2 is required for chromosome replication in diploid cells and chorion gene amplification in anuploid ovarian follicle cells (6, 19). These observations suggest an evolutionary conserved role for ORC in DNA replication.

In addition to its role in initiation of DNA replication in *S. cerevisiae,* ORC has separable functions in S and M phase (20) and an interesting function in transcriptional silencing of the mating type loci (21–27). ORC binds to specific, *cis*-acting silencer elements that are adjacent to the *HMR***a** and *HML*α silent mating-type genes (21–24, 28). To facilitate this function, the budding yeast silencing protein Sir1p has been shown to associate with ORC via an interaction with Orc1p (29, 30). Sir1p has been proposed to stabilize a heterochromatin-like protein complex containing ORC, Rap1p, Sir2p, Sir3p, and Sir4p, which represses gene expression in a heritable manner (31).

In *Drosophila*, transcriptional silencing and position effect variegation of gene expression occurs when chromosomal rearrangements bring genes in close proximity to heterochromatin (32). This silencing requires the participation of a structural component of heterochromatin known as HP1 (33). Interestingly, *Drosophila* ORC (dORC) binds to HP1, and heterozygous, recessive lethal mutations in *ORC2* cause a suppression of position effect variegation and disrupted localization of HP1 (34, 35). These findings suggest that ORC plays a role in recruitment of silencing factors to specific chromosomal loci in both yeast and *Drosophila*. It has not been determined, however, whether ORC has a similar function in mammalian cells.

During a search for factors that bind to human ORC protein subunits, we uncovered a novel protein that interacts with the largest subunit of human ORC, ORC1. A full-length cDNA encoding the novel protein was cloned, revealing sequence similarities to a subfamily of recently identified histone acetyltransferases that contain the MYST domain, including the *S. cerevisiae* Sas2p. The protein encoded by this cDNA was termed Histone acetyltransferase Bound to ORC 1 (HBO1). Biochemical studies show that a fraction of the HBO1 protein binds to ORC1 *in vivo* and that HBO1 is in a large protein complex that contains histone acetyltransferase activity. The biochemical interaction between human ORC1 and HBO1 proteins suggest similarities to the genetic interactions between *S. cerevisiae* Orc1p and a putative histone acetyltransferase called Sas2p, a protein involved in transcriptional silencing in yeast.

EXPERIMENTAL PROCEDURES

Identification of a cDNA Encoding a Human ORC1 Protein-binding Protein—Human full-length ORC1 cDNA from pKG28 (11) was cloned into the *Sma*I and *Bam*HI sites of the pBTM116 vector (a gift from Linda van Aelst, Cold Spring Harbor Laboratory), carrying the *TRP1* gene and a lexA DNA-binding domain, to generate plexAhORC1. Yeast strain L40 (36, 37) (*MAT* **a** *his3*Δ200 trp1–901 leu2–3, 112 ade2 lys2–801am URA3::(lexAop)8-*lacZ LYS2*::(lexAop)8-*HIS3*) harboring the plexAhORC1 plasmid was transformed with a cDNA library cloned in the pGADGH vector (a gift from Linda van Aelst), carrying the *LEU2* gene and the GAL4 activation domain. 3×10^6 transformants were screened initially by histidine nutritional selection and 975 His^+ colonies were assayed for β-galactosidase activity, resulting in 314 His^+ β-gal^+ clones. 171 Trp^- Leu^+ clones, which lost β-galactosidase activity after curing of the bait plasmid plexAhORC1 were selected. Restoration of β-galactosidase activity was assayed to test the specificity of interaction after adding back the plasmid plexAhORC1 or plexAHRAS by mating with AMR70 (*MAT***a** *his3*Δ*200 lys2–801am trp1–901 leu2–3 112 URA*::

* The research was supported by Grant CA13106 from the NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF074606.

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[1] The abbreviations used are: ORC, origin recognition complex; SAS, something about silencing; HAT, histone acetyltransferase; MYST, a protein domain found in HATs; HBO, histone acetyltransferase bound to ORC; GST, glutathione *S*-transferase.

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(lexAop)8-lacZ) (38) harboring that plasmid. cDNA clones encoding putative ORC1-interacting proteins were rescued from yeast, transformed into *Escherichia coli* DH5α, isolated, and sequenced on both strands with the use of an ABI 377 sequencer. The 5′ region of the HBO1 cDNA was cloned (pL74w1) by hemi-nested polymerase chain reaction with a λgt10 vector primer (5′-CTTATGAGTATTTCTTCCAGGGTA-3′) and gene-specific primers (5′-CACCAGGTGGGTGTTTCCACACAC-3′, 5′-CACCTTGCATTCGTCAGCTGAGAA-3′, and 5′-CGCGTCGACCTGCCTGAGTCCTTCAGGGA-3′) using as template DNA, a cDNA library from the human teratocarcinoma cell line NTera2D1 (39). The nucleotide sequence of the cloned polymerase chain reaction product was verified by comparison to that determined by direct sequencing. A full-length cDNA clone was constructed (plasmid pBSSKL74). The GenBank™ accession number is AF074606. A comparison of the human cDNA against the National Center for Biotechnology Information (NCBI) data bases was done with the basic local alignment search tool (BLAST) algorithm (40).

The open reading frame encoding the HBO1 protein was fused at its 5′ end to the open reading frame encoding glutathione *S*-transferase (GST) in the vector pET11cGST (a gift from Masumi Hidaka, National Institute of Basic Biology, Japan) to create plasmid pGSTL74. The recombinant protein was expressed in *E. coli* and purified by column chromatography on glutathionine-Sepharose-4B as described by the manufacturer (Amersham Pharmacia Biotech).

RNA Expression—Multiple tissue Northern blots (CLONTECH) were probed using the Express Hybridization solution (CLONTECH) with a ^{32}P-labeled cDNA probe (pL74w1) prepared using the Prime-It random primer labeling kit (Stratagene) and a Microspin S-400 HR Column (Amersham Pharmacia Biotech) to remove unincorporated [α-^{32}P]dCTP.

Antibodies and Immunological Methods—Peptides CSH429 (H-MAHYPTRLKTRKTY-NH_2) derived from the human ORC1 peptide sequence (11) and L74-1 (H-SLKDSGSDLSHRPKR-NH_2) derived from the HBO1 peptide sequence (Fig. 1*A*) were synthesized (Research Genetics), conjugated to keyhole limpet hemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (Pierce) and injected into rabbits to generate polyclonal anti-peptide antisera (named CS171 for hORC1, and CS445 and CS446 for HBO1). Antibodies were affinity purified as described (41) using a peptide affinity column prepared by cross-linking the peptide to Sulfo Link resin (Pierce). The hORC1 and HBO1 cDNAs were cloned into the *Bam*HI and *Xba*I sites of the NCITE vector (a gift from William Tansey and Winship Herr, Cold Spring Harbor Laboratory), a derivative of the pCITE-2a(+) vector (Novagen) with a hemagglutinin epitope tag to create the plasmid pNCITEL74. *In vitro* translation was performed using the TNT system (Promega) and the products were diluted 25-fold in buffer H/0.05 (20 mM HEPES-NaOH (pH 7.5), 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, 10% glycerol, 0.01% Nonidet P-40; containing 50 mM NaCl) and incubated at 4 °C for 2 h before immunoprecipitation with rabbit anti-hORC1 (CS171) or rabbit anti-HBO1 (CS446) crude serum and protein A-Sepharose beads. Following extensive washes in buffer H/0.05, immunoprecipitated proteins were detected by SDS-polyacrylamide gel electrophoresis and fluorography.

293 cell nuclear extract (42) was immunoprecipitated in buffer H/0.2 with anti-hORC1 antibodies (CS171) cross-linked to protein A-Sepharose beads (Amersham Pharmacia Biotech). After extensive washes with buffer H/0.2, immunoprecipitated proteins were eluted and analyzed by Western blotting and probed with anti-HBO1 (CS445) antibodies. For transient expression of human ORC1 tagged with the phage T7 epitope (T7-hORC1), 293 cells were electroporated with 10 μg of pKG28 (11), and cell extracts were prepared as described previously (11). The soluble lysate was subjected to immunoprecipitation with affinity purified antibodies against HBO1 protein cross-linked to protein A-Sepharose beads (Amersham Pharmacia Biotech). The immune complexes were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with a mouse monoclonal antibody against the T7 epitope (Novagen).

Mapping the Interaction Site within Human ORC1—plexAhORC1cas was constructed by inserting the cassette 5′-*Bam*HI-CTACCGCGGTAACTGACTGG-*Sal*I-3′, at the 3′ end of the coding sequence of plexAhORC1, to ensure efficient termination of translation. Plasmids plexAhORC1 (1–621) and plexAhORC1 (1–208) were generated by digestion of plexAhORC1cas with *Sac*I plus *Bam*HI and *Msc*I plus *Bam*HI, respectively, followed by end filling with the Klenow enzyme, gel purification of the larger DNA fragment, and self-ligation. A 6-kilobase pair DNA fragment released by *Eco*RI digestion of plexAhORC1cas was gel purified and self-circularized to produce plexAhORC1 (510–861). A 0.6-kilobase pair *Eco*RI-*Bgl*II fragment of plexAhORC1cas was ligated to the 5-kilobase pair *Eco*RI-*Bam*HI fragment of plexAhORC1cas to generate plexAhORC1 (1–278). A 2-kilobase pair fragment derived from *Msc*I digestion and Klenow treatment followed by *Bam*HI digestion of plexAhORC1cas was inserted into the *Sma*I and *Bam*HI sites of the pBTM116 vector to generate plexAhORC1 (210–861). In-frame fusion of lexA and hORC1 in the plexAhORC1 (210–861) construct was confirmed by sequencing. A DNA fragment encoding hORC1 (488–750) was amplified from plexAhORC1 (488–750) by polymerase chain reaction using primers 5′-CCGGGATCCTGGAGGAAGCCCGACTGA-3′ and 5′-CGCGTCGACTTAGTGGGCTATGGTGACCAGGC-3′ and *Pfu* DNA polymerase (Stratagene), cut with *Sal*I and *Bam*HI, and cloned into the *Sal*I and *Bam*HI sites of pBTM116 to generate plexAhORC1 (488–750), whose sequence was verified by DNA sequencing.

Nucleosome Reconstitution—Nuclear histones were reconstituted using recombinant *Xenopus* $H3_2$·$H4_2$ tetramers (8 μg) (a gift from Drs. K. Luger and T. Richmond, Eidgenössische Technische Hochschule, Zürich) and a plasmid pEGFP-C3 (CLONTECH) (15 μg) by salt gradient dialysis (43).

Detection of Histone Acetyltransferase Activity—The HBO1 polypeptide was immunoprecipitated with anti-HBO1 serum from a 293 nuclear extract (42) in buffer H/0.2. Histone acetyltransferase (HAT) activity was determined with a mixture of recombinant *Xenopus* histone $H3_2$·$H4_2$ tetramers (100 μg/ml) (a gift from Drs. K. Luger and T. Richmond) (44), human histone H2A·H2B purified from HeLa cells (100 μg/ml) (a gift from Dr. A. Verreault) (45), and 23 pmol of [^{3}H]acetyl coenzyme A (11.2 Ci/mmol, Andotek) in 12.5 μl of HAT buffer (25 mM Tris-HCl, pH 8.5, 1 mM dithiothreitol, 0.5 mM EDTA, 5 mM sodium butyrate, 150 mM NaCl, 10% glycerol) and incubated at 37 °C for 1 h. The histones were resolved in a SDS-18% polyacrylamide gel (46), and visualized by Coomassie Brilliant Blue staining and fluorography. The human HAT1 holoenzyme (a gift from Dr. A. Verreault) was used as a positive control in HAT assays. For quantitative liquid assays, HAT activity was measured after incubation at 37 °C for 30 min using a phosphocellulose filter-binding assay, as described previously (47).

Affinity Purification of HBO1 Protein—HBO1 was immunoprecipitated from a 293 cell nuclear extract (42) in buffer H/0.4 with affinity purified anti-HBO antibodies (CS446) cross-linked to protein A-Sepharose beads. After extensive washing with buffer H/0.4, the beads were incubated at 4 °C for 30 min. in buffer H/0.4 containing 2 mg/ml of the L74–1 peptide to elute the immune complex.

A 293 cell nuclear extract was dialyzed against buffer H/0.1 and loaded onto a 0.25-ml POROS 20 HQ anion exchange column (Roche Molecular Biochemicals). The column was eluted at 0.2 ml/min with a linear salt gradient (2.5-ml gradient) from 100 to 1,000 mM NaCl in buffer H.

RESULTS AND DISCUSSION

To gain insight into human ORC function, we screened a HeLa cell cDNA library by the two-hybrid method using human ORC1 protein (hORC1) as bait, and obtained eight independent cDNA clones whose interaction with hORC1 was specific. The characterization of one clone, L74, is described here. The cDNA sequence encodes a 611-amino acid protein with a predicted molecular mass of 83 kDa (Fig. 1*A*). No in-frame stop codon was found upstream of the first methionine, however, this methionine is likely to be the initiation codon, because the size of the protein translated from this cDNA is the same as that of the protein present in human cells (data not shown).

Based upon our finding that the L74 cDNA encodes a protein that binds to hORC1 and is a subunit of a histone acetyltransferase (see below), we termed the protein HBO1, an acronym for Histone acetyltransferase Binding to hORC1. Two sequence motifs were noted (Fig. 1*B*). One was a serine-rich region located at the amino terminus of the protein. The other feature, revealed by a BLASTP search of the nonredundant protein data base, is a 270-amino acid carboxyl-terminal region known as a MYST domain (48–52). This motif contains a putative acetyl-coenzyme A (acetyl-CoA) binding domain (53) (Fig. 1*C*). Throughout the MYST domain, HBO1 is approximately 50% identical and 70% similar to several other MYST domain proteins (Fig. 1*B*), many of which have been implicated in transcriptional regulation. One particular protein of interest, Sas2p, promotes silencing of the silent mating type gene at

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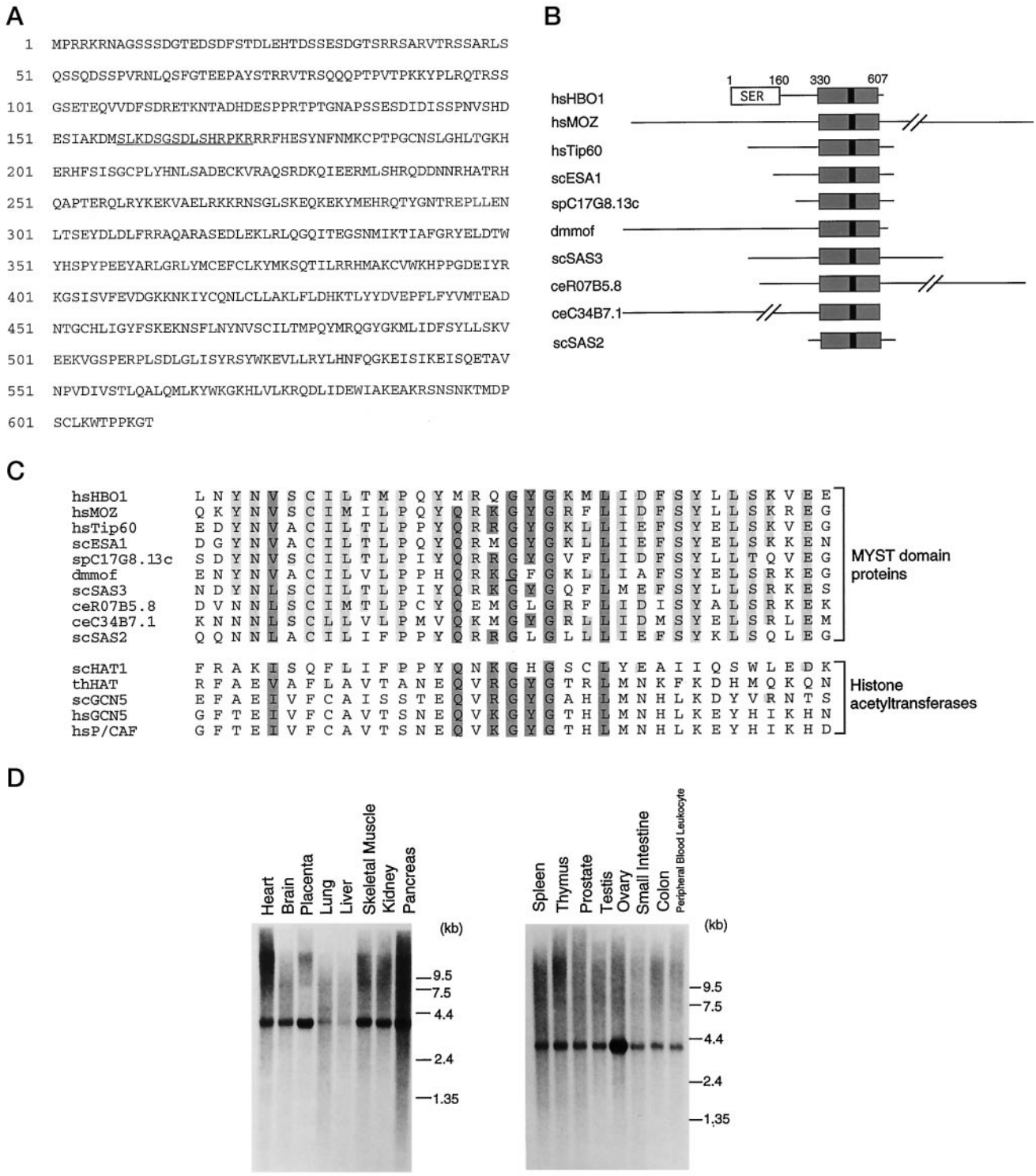

FIG. 1. **Amino acid sequence of HBO1, comparison with other MYST domain proteins, and mRNA expression in human tissues.** *A*, amino acid sequence of HBO1 deduced from the cDNA clone. The underlined peptide sequence was used as an antigen to generate a rabbit polyclonal antiserum against HBO1. *B*, diagram showing the relationship between HBO1 and other MYST domain proteins. The MYST domain itself is represented as a *gray box*, and the putative acetyl-CoA-binding domain is shown as a black box. The serine-rich domain is depicted as SER. The MYST domain proteins are listed in decreasing order of similarity with HBO1 within the MYST domain. Abbreviations are as follows: *Moz*, monocytic leukemia zinc finger protein (51); *Tip60*, Tat interactive protein, 60 kDa (16); *SAS*, something about silencing (48, 49); *ESA*, essential SAS-related acetyltransferase (54); *mof*, males-absent on the first (50); *hs*, *Homo sapiens*; *sc*, *S. cerevisiae*; *sp*, *S. pombe*; *dm*, *D. melanogaster*; *ce*, *Caenorhabditis elegans*; AC17G8.13c (accession number Z69795), R07B5.8 (accession number Z72512), and C34B7.1 (accession number Z83220) are open reading frames derived from various genome sequencing projects. *C*, amino acid sequence alignments of motif A among various members of the GCN5-related *N*-acetyltransferase (GNAT) superfamily (53) (the Tip60 sequence is from Yamamoto and Horikoshi, see Ref. 55). *Dark shading* indicates similar amino acids that are shared by more than six MYST domain proteins and more than three histone acetyltransferases, whereas *pale shading* denotes similar amino acids that are shared by more than six MYST domain proteins but fewer than three histone acetyltransferases. The *underlined* G residue is the mutated amino acid in the *mof* mutant defective in dosage compensation (50). *D*, Northern blots were probed with a ^{32}P-labeled 500-base pair fragment encoding the NH_2-terminal portion of HBO1. The position of RNA size markers is also shown. A probe of these blots with an actin probe revealed relatively constant RNA levels in these blots (data not shown).

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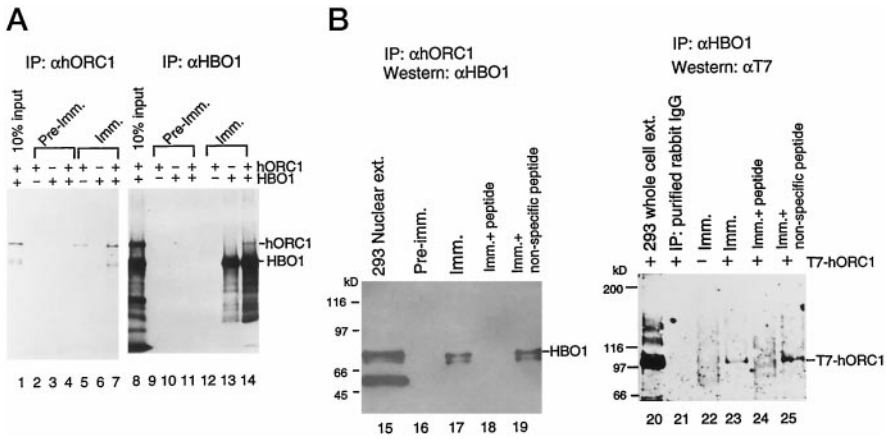

FIG. 2. **Association between hORC1 and HBO1 in the rabbit reticulocyte lysate and in human 293 cell extracts.** *A*, hORC1 and HBO1 interaction in the rabbit reticulocyte lysate. hORC1 and HBO1 were translated *in vitro* in the rabbit reticulocyte lysate and immunoprecipitated with either a rabbit anti-hORC1 serum (*left panel*) or a rabbit anti-HBO1 serum (*right panel*). hORC1 alone was expressed in *lanes 2*, *5*, *9*, and *12*, whereas HBO1 alone was expressed in *lanes 3*, *6*, *10*, and *13*. Both proteins were co-translated in *lanes 4*, *7*, *11*, and *14*. One-tenth of the input proteins was loaded in *lanes 1* (hORC1) and *8* (HBO1) to give an estimate of the efficiency of immunoprecipitation. *B*, hORC1 and HBO1 interact in human cell extracts. *Left*, following immunoprecipitation from 293 cell nuclear extracts with affinity-purified anti-hORC1 antibodies (CS171), co-precipitated proteins were Western blotted and probed with anti-HBO1 antibodies. 293 cell nuclear extract (20% of input, *lane 15*), immunoprecipitate using pre-immune serum (*lane 16*), immune serum (*lane 17*), immune serum plus antigenic peptide (*lane 18*), and immune serum plus nonspecific peptide (*lane 19*). *Right*, 293 cells were electroporated with 10 μg of a plasmid for transient expression of T7 epitope-tagged hORC1. Whole cell lysate from T7-ORC1-transfected 293 cells (*lane 20*), immunoprecipitate from transfected cells with purified rabbit IgG (*lane 21*), immunoprecipitate from mock-transfected cells with anti-HBO1 antibodies (*lane 22*), immunoprecipitates from transfected cells with anti-HBO1 antibodies (*lane 23*), anti-HBO1 antibodies plus antigenic peptide L74–1 (*lane 24*), and anti-HBO1 antibodies plus nonspecific peptide (*lane 25*) were subjected to Western blotting analysis and probed with a mouse monoclonal antibody against the T7 epitope tag.

HMLα and genes placed near a telomere (48). In contrast, Sas2p (and other *SAS* gene products) antagonizes silencing at the *HMR***a** locus when the essential *HMR*-E silencer element is mutated. Interestingly, the latter function of Sas2p is mediated through ORC (49).

Of the other MYST domain proteins, the *Drosophila* "males-absent on the first" (*mof*) gene has been shown to be involved in dosage compensation, a process that results in a two-fold increase in transcription of the single X chromosome in male flies and is accompanied by histone H4 hyperacetylation at lysine 16 (50). Interestingly, the *mof* mutant in which both dosage compensation and H4 acetylation at position 16 are reduced has a single amino acid substitution from Gly to Glu at position 691. This glycine residue is absolutely conserved among the putative acetyl-CoA-binding motifs of the various MYST domain family members and other histone acetyltransferases (Fig. 1*C*). The human MOZ gene is rearranged and fused to the gene encoding the CREB-binding protein in a recurrent chromosomal translocation characteristic of acute monocytic leukemia (51). Tip60 interacts with the human immunodeficiency virus Tat protein and augments expression from the HIV-1 promoter (52).

Northern blot analysis revealed that the HBO1 mRNA was expressed in all human tissues tested (Fig. 1*D*). HBO1 mRNA abundance was not strictly correlated with cell proliferation and was particularly high in ovarian tissue.

To test the interaction between hORC1 and HBO1 by an independent approach, *in vitro* translated hORC1 and HBO1 proteins were subjected to co-immunoprecipitation assays. As shown in Fig. 2*A* (*left panel*), polyclonal anti-hORC1 serum co-immunoprecipitated the HBO1 polypeptide only in the presence of co-translated hORC1 (*lanes 5-7*). In a reciprocal experiment, immunoprecipitation with polyclonal anti-HBO1 serum (Fig. 2*A*, *right*) co-precipitated the hORC1 polypeptide only in the presence of co-translated HBO1 protein (*lanes 12–14*). In both cases, neither hORC1 nor HBO1 was immunoprecipitated by pre-immune sera (*lanes 2–4* and *lanes 9–11*). These results confirm the existence of an interaction between hORC1 and HBO1, although the possibility remained that protein(s) in the rabbit reticulocyte lysate may mediate this interaction.

An association between HBO1 and hORC1 in human cells was tested. Nuclear extracts from 293 cells were immunoprecipitated with polyclonal anti-hORC1 serum and co-precipitation of the HBO1 polypeptide was assayed by Western blotting analysis. The HBO1 polypeptide could be co-immunoprecipitated only with immune anti-hORC1 serum (Fig. 2*B*, *lanes 16* and *17*), and co-immunoprecipitation was blocked by addition of excess antigenic peptide (*lane 18*), but not by a nonspecific peptide (*lane 19*). Because 20% of the nuclear extract used for the immunoprecipitations was loaded in *lane 15*, we conclude that only a small portion of the HBO1 protein associated with hORC1 in human cells (Fig. 2*B*, *left panel*).

Although we could readily detect co-immunoprecipitation of the HBO1 polypeptide with anti-hORC1 antibodies, the reciprocal experiment did not work even under mild washing conditions. This was most likely due to epitope masking in the HBO1-hORC1 complex or to the small portion of HBO1 bound to hORC1, coupled with the fact that hORC1 exits as a relatively low abundance protein in human cell extracts (data not shown). To circumvent these problems, extracts from 293 cells transiently expressing T7 epitope-tagged hORC1 were prepared for immunoprecipitation with affinity purified anti-HBO1 antibodies. This approach did reveal co-immunoprecipitation of T7 epitope-tagged hORC1 (Fig. 2*B*, *lane 23*), that was blocked by addition of excess antigenic peptide (*lane 24*), but not by addition of a nonspecific peptide (*lane 25*). No HBO1 signal was detected in negative control reactions performed either by immunoprecipitation from 293 cell extracts containing T7 epitope-tagged hORC1 with purified rabbit IgG (*lane 21*) or by immunoprecipitation from mock-transfected 293 cell extracts with anti-HBO1 antibodies (*lane 22*).

We sought to determine whether HBO1 bound to the multisubunit ORC protein rather than to hORC1 alone. Unfortunately, the only other human ORC subunit against which antibodies were currently available is hORC2 (11). We have been unable to co-immunoprecipitate hORC2 and HBO1 from human 293 cell nuclear extracts or from 293 cells overexpressing the T7-hORC1 polypeptide (data not shown), although we know

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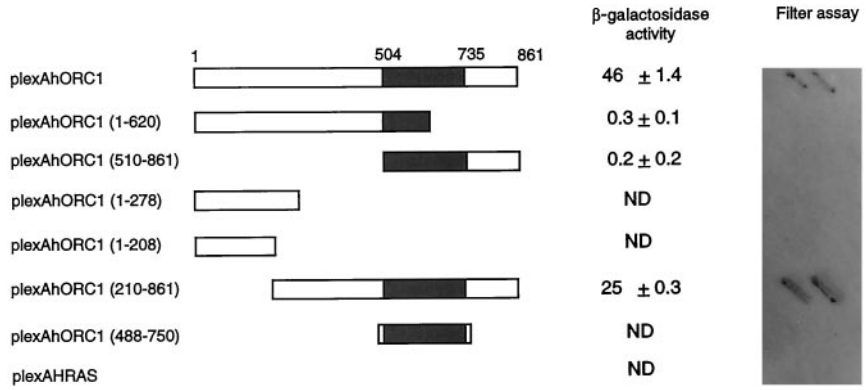

FIG. 3. **Mapping of hORC1 region required for binding to HBO1.** hORC1 deletion mutants fused to the lexA DNA-binding domain (lexA) were individually co-transformed into yeast strain L40 along with a construct expressing the GAL4 transcriptional activation domain fused to the ORC1-binding domain of HBO1 (amino acid residues 158–611). Transformants were grown in selective medium and β-galactosidase activity was measured using both a quantitative liquid assay and a filter assay. The values shown are the mean ± S.D. of three independent quantitative liquid assays. *ND*, not detectable in the liquid assay. The *gray box* represents the cell division cycle-nucleoside triphosphate (CDC-NTP) region conserved among the Oc1p- and Cdc6p-related proteins (11, 60).

that the ectopically expressed T7-ORC1 did not efficiently associate with endogenous ORC2. In addition to the other ORC subunits, the two ORC1-interacting proteins that have been isolated play a role in heterochromatin function. The budding yeast silencing protein Sir1p has been shown to bind to the NH_2-terminal portion (amino acid residues 5–228) of *S. cerevisiae* Orc1p (29). Similarly, *Drosophila* HP1 also binds to the NH_2-terminal domain of dORC1 (amino acid residues 161–319) (35). Thus, the NH_2-terminal domain of ORC1, although it is not structurally conserved through evolution, may have a generally conserved function in recruitment of heterochromatin proteins. To map the region of hORC1 responsible for binding to HBO1, a series of hORC1 deletion mutants fused to the lexA DNA-binding domain were constructed, co-transformed into yeast L40 cells with a plasmid encoding HBO1 that was fused to the GAL4 transcriptional activation domain, and the transformants were tested for β-galactosidase activity (Fig. 3). Of six ORC1 deletion mutants tested, only the mutant carrying amino acid residues 210–861 of hORC1 was positive for β-galactosidase activity. In the two-hybrid assay, the NH_2-terminal portion of hORC1 (1–208) was found to be dispensable for binding to HBO1, consistent with the finding that anti-hORC1 antibodies (CS171), which recognize an NH_2-terminal peptide of hORC1, did not disrupt the interaction between hORC1 and HBO1 in the co-immunoprecipitation experiments using *in vitro* translated proteins (Fig. 2*A*).

Based upon the presence of a putative acetyl-CoA binding motif shared by many acetyltransferases (Fig. 1*C*) and the finding that the *Drosophila mof* mutant displays reduced acetylation of histone H4 at lysine 16 on the male X chromosome (50), it has been proposed that MYST domain proteins may generally act as HATs. Recently, both human Tip60 and yeast Esa1p proteins were shown to be histone H3 and H4 acetyltransferases (54–56). To test whether HBO1 might have HAT activity, the protein was immunoprecipitated from 293 cell nuclear extracts and assayed for HAT activity using recombinant core histones as substrates. Immunoprecipitates from 293 cell nuclear extracts with polyclonal anti-HBO1 antibodies had an activity capable of acetylating histones H3 and H4 and, more weakly, H2A (Fig. 4*A*, *lane 4*), suggesting that the immune complex containing the HBO1 protein had HAT activity. In contrast, pre-immune serum did not immunoprecipitate any HAT activity (Fig. 4*A*, *lane 3*). In this experiment, human HAT1, a histone H4 (and H2A) specific HAT was used as a control (45).

To test whether any of these HAT activities were associated with HBO1 in a salt-resistant manner, we immunoprecipitated 293 nuclear extract with anti-HBO1 antibodies and washed the beads in buffers containing increasing concentrations of sodium chloride, followed by HAT assays. The H2A HAT activity was eluted at relatively low salt concentration (less than 0.2 M sodium chloride, Fig. 4*B*, *lanes 7* and *8*), suggesting that this activity may be due to nonspecific adsorption of an H2A acetyltransferase to the HBO1 antibody beads. In contrast, most of the H3 and H4 HAT activities remained bound to the HBO1 antibody beads up to at least 0.8 M sodium chloride (Fig. 4*B*, *lanes 10-12*), suggesting that these activities were either due to HBO1 itself or were mediated by polypeptides that were associated with HBO1 in a highly salt-resistant manner. Under all these conditions, the HBO1 polypeptide remained quantitatively bound to the antibody beads, as determined by Western blotting (data not shown). To determine whether additional polypeptides were associated with HBO1, HBO1 was eluted from the immune complex by addition of excess antigenic peptide. The eluted fraction was assayed for the presence of HBO1 by immunoblotting, HAT activity, and its polypeptide composition determined by SDS-polyacrylamide gel electrophoresis and silver staining. The HBO1 polypeptide (Fig. 4*B*, *lane 16*) and the histone H3 and H4 HAT activities (*lane 19*) were all present in the eluate from the HBO1 antibody beads. Several additional polypeptides co-eluted with HBO1 (Fig. 4*B*, *lane 14*). This result suggests that either HBO1 itself or HBO1 and a tightly associated protein acetylates H3 and H4.

To provide further support for the existence of a HBO1 protein complex, 293 cell nuclear extracts were fractionated by anion exchange chromatography, and the column fractions were directly probed by Western blotting with anti-HBO1 antibodies or subjected to immunoprecipitation with anti-HBO1 antibodies, followed by HAT activity assays (Fig. 4*D*). This fractionation procedure produced a peak of HBO1 protein (from fractions 26 through 41) (Fig. 4*D*, *upper panel*). When immunoprecipitated with HBO1 antibodies, only the column fractions coinciding with the peak of HBO1 polypeptide had HAT activity (Fig. 4*D*, *lower panel*). The two peaks may represent two separate complexes containing HBO1 or more likely, they are due to the assay of HBO1 activity in immunoprecipitates. Nevertheless, the HAT activity does co-fractionate with the HBO1 protein. In addition, using cation exchange chromatography on a POROS 20 HS column, a peak of HAT activity was also observed to co-purify with HBO1 when fractions containing HBO1 were immunoprecipitated with HBO1 antibodies, followed by HAT assays (data not shown). These co-fractionation data strongly suggest that either HBO1 itself or a protein tightly bound to HBO1 has HAT activity.

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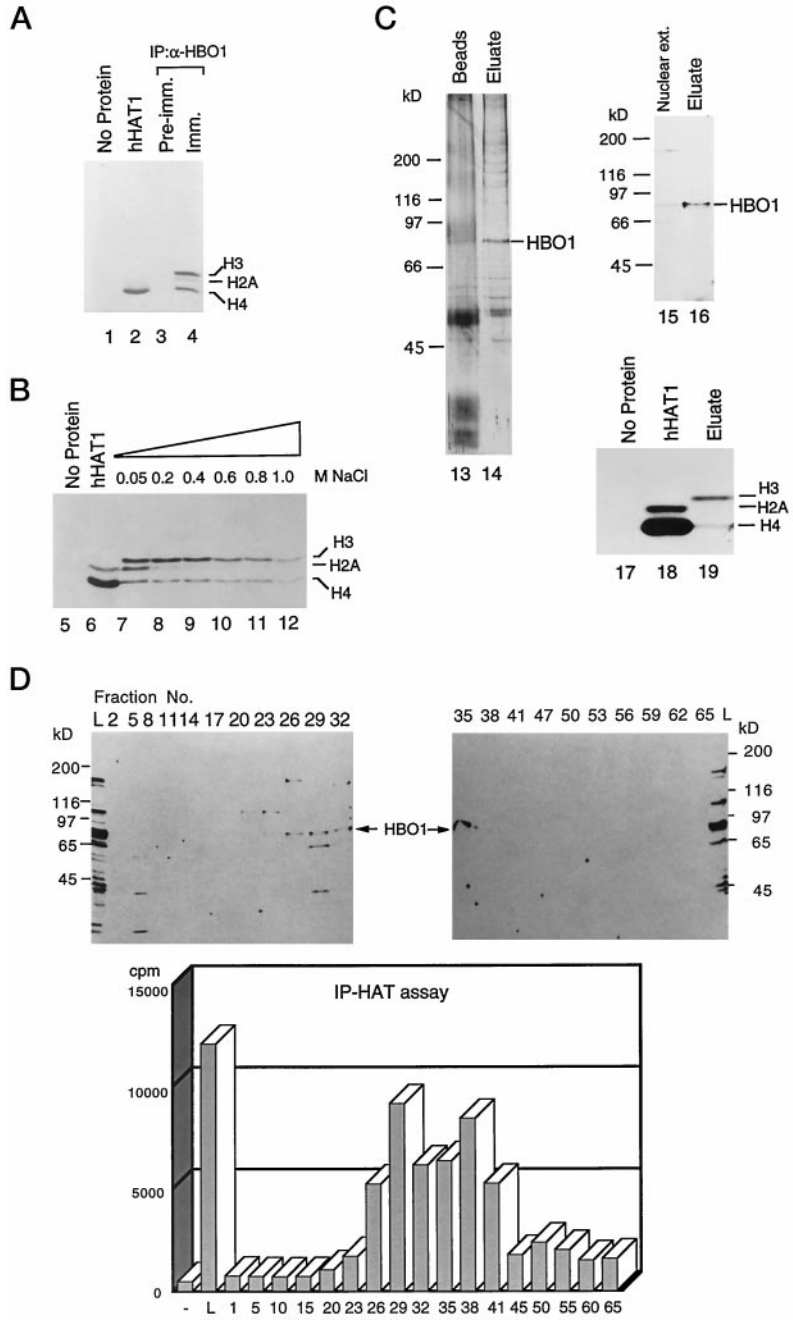

FIG. 4. **Histone acetyltransferase activity of the HBO1 complex.** A protein complex that includes HBO1 has histone acetyltransferase activity when isolated from human cells by immunoaffinity purification. *A*, proteins from a 293 cell nuclear extract were immunoprecipitated with either pre-immune serum (*lane 3*) or anti-HBO1 serum (*lane 4*). Controls for HAT assays were performed without protein added (*lane 1*) or with human HAT1 holoenzyme (*lane 2*). *B,* HBO1 immunoprecipitated from 293 cell nuclear extract was washed with buffers of increasing salt concentration, then assayed for HAT activity. *C*, HBO1 immunoprecipitated from 293 cell nuclear extract in 0.4 M NaCl with affinity purified anti-HBO1 antibodies (CS446) cross-linked to protein A-Sepharose was eluted by addition of excess antigenic peptide. Eluates were separated by SDS-polyacrylamide gel electrophoresis followed by silver staining, assayed for HBO1 protein by Western blotting analysis, and also assayed for histone acetyltransferase activity. *D*, a 293 cell nuclear extract was fractionated by anion exchange chromatography. Fractions were probed with anti-HBO1 antibody (*top*) and subjected to immunoprecipitation with HBO1 antibodies followed by HAT assays (*bottom*). *L*, column load; —, no protein control.

We also tried to determine whether HBO1 has an intrinsic HAT activity by expressing recombinant HBO1 protein as a GST fusion protein in *E. coli*. This protein had no detectable HAT activity under a variety of pH (6.5–9.0) and divalent zinc ion concentrations (1 μM-50 mM) (data not shown). (We reasoned that the HAT activity of MYST domain proteins may be zinc-dependent because this domain commonly contains a C2HC-type zinc finger.) One possibility is that HBO1 indeed has intrinsic HAT activity but the recombinant protein expressed *in E. coli* either failed to fold into a proper three-dimensional structure or lacked a post-translational modification such as phosphorylation, resulting in a lack of HAT activity. However, it is more likely that additional polypeptides associated with HBO1 in human cells were required for HAT activity. For instance, the HAT activity of *S. cerevisiae* and human HAT1 are stimulated by association with a core histone-binding subunit known as HAT2 and the transcriptional activator GCN5 only acetylates nucleosomes efficiently when the GCN5 catalytic subunit is part of a large multisubunit complex (45, 57).

To test whether HBO1 alone or as part of a protein complex was capable of acetylating nucleosomes, HAT activity was assayed using reconstituted nuclear histones ($H3_2{\cdot}H4_2$ tetramers) as substrates. Human Hat1 holoenzyme used as a control acetylated only free histones (Fig. 5, *lane 6*) but not nucleosomal substrates (Fig. 5, *lane 1*) as previously reported (45). In contrast, the protein complex containing HBO1 acetylated nuclear histones relatively weakly (Fig. 5, *lane 2*) compared with free histones (*lane 5*). The histone acetyltransferase activity using different substrates required different optimal assay conditions, therefore there was a stronger signal using the free histones (Fig. 5, *lane 5*) than with histones present in nucleosomes (Fig. 5, *lane 2*). The recombinant GST-HBO1 fusion protein acetylated nuclear histones very weakly, and the labeled signal was only visible after longer exposure (Fig. 5, *lane 4* and data not shown), whereas GST alone had no HAT activity

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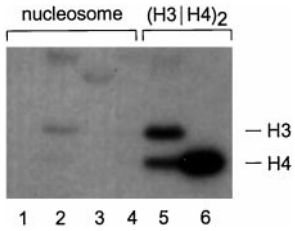

FIG. 5. **HBO1 activity on free histones H3.H4 and nucleosomal histones.** Histones H3.H4 tetramers (*lanes 5* and *6*) and nucleosomal chromatin was reconstituted by salt gradient dialysis into a nucleosomal-like structure (*lanes 1–4*) and were tested as substrates for histone acetyltransferase activity using various forms of HBO1. As judged by Coomassie Brilliant Blue staining, equal amounts of histones H3 and H4 were used in all the assays. The human HAT1 enzyme (45), which acetylates only free histones, was used as a control in *lanes 1* and *6*. HBO1 protein complex was purified from human placental extract by antibody affinity chromatography (*lanes 2* and *5*), recombinant GST-HBO1 (*lane 4*), or GST (*lane 3*) were the sources of the HBO1 protein. The acetyltransferase activity was measured as described under "Experimental Procedures."

(Fig. 5, *lane 3*) regardless of the exposure time. Thus HBO1 had a very weak intrinsic HAT activity using the nucleosome histones, but not free histones. Because HBO1 (pI = 9.1) and histones are both basic proteins, HBO1 protein may not readily access free histone substrates in the HAT reaction due to mutual electrostatic repulsion. On the other hand, when nucleosomal histones were used in the HAT assay, the positive charge of histones was neutralized by deposition onto DNA, and this may facilitate access by HBO1 protein, resulting in the weak positive HAT activity. But it is clear that under the conditions we tested, the native HBO1 protein complex had substantially higher HAT activity than the recombinant GST-HBO1 protein.

Histone acetylation has been generally implicated in transcriptional activation (58). Thus, one obvious possibility from our studies is that HBO1 acetylation of histones or another protein may activate DNA replication. This could facilitate access to DNA replication initiation sites for DNA replication proteins or plasticity of nucleosomes as DNA replication occurs. Alternatively, because MYST domain proteins have been shown to be involved in transcriptional silencing (Sas2p and Sas3p) (48), dosage compensation (*mof*) (50), and transcriptional activation (Tip60) (52), HBO1 may play a role in the control of gene expression mediated by the interaction with hORC1. We note that *Drosophila* ORC has been implicated in position effect variegation of gene expression and heterochromatin function (35). It may be that ORC bound to heterochromatin could facilitate specific histone acetylation patterns, thereby insuring the silencing of gene transcription in these regions of the chromosomes.

In this regard, genetic studies with *SAS2* in *S. cerevisiae* suggest that this HBO1-related MYST domain protein antagonizes the function of ORC in DNA replication and effects ORC-mediated transcriptional silencing of the mating-type genes (49). Disruption of the *SAS2* gene partially suppresses the temperature sensitivity of *orc2–1* and *orc5–1* mutants. Sas2p also antagonizes ORC mediated silencing at an altered HMR silencer, but helps silencing at HML (49). Sas2p may exert such functions by acetylation of histones, by acetylation of one of the ORC subunits, or more simply, by binding to ORC. It is not yet clear whether SAS proteins bind directly to Orc1p in *S. cerevisiae*. None of the three budding yeast Sas2p-like proteins (Sas2p, Sas3p, and Esa1p) that we tested bound to *S. cerevisiae* Orc1p in two-hybrid assays (data not shown).

MYST domain proteins have been proposed to regulate transcription through protein acetylation (48–51), although this has not been shown directly for any member of this family. Among the various members of the GCN5-related N^{ϵ}-acetyltransferase superfamily, GCN5-related HATs, P/CAF, Hat1, and GCN5 all contain three amino acid sequence motifs, named A, B, and D (53). In contrast, MYST domain proteins only have motif A. Because HBO1 protein is a MYST domain protein and protein complexes containing the HBO1 exhibit histone acetyltransferase activity, we suggest that HBO1 is an acetyltransferase. Unlike Esa1p (54, 56), HBO1 seems to require other as yet unidentified stimulatory subunits. Sequence comparisons show that of the yeast MYST domain proteins, Esa1p is closest in primary sequence to human HBO1, but we caution that interpretation of functional homology based on sequence similarity within a family of proteins is risky. For example, comparison of the primary sequences between the human and yeast WD40-repeat, histone-binding proteins did not predict functional homologues (45). In contrast, genetic interactions between the genes encoding Sas2p and the ORC subunits suggest that HBO1 might be functionally related to Sas2p protein.

Recently, Gu and Roeder (59) reported that p53 is acetylated by p300, a known histone acetyltransferase and transcriptional coactivator that is physically associated with p53. Acetylation of p53 by p300 stimulated sequence-specific DNA binding by p53 (59). Regulation of DNA binding by transcription factor acetylation therefore represents a potentially novel mechanism of transcriptional activation. By analogy, replication proteins such as ORC may be regulated by HBO1-mediated acetylation.

Acknowledgments—We thank Erica Golemis for the lexA antibody, Linda van Aelst, Kyoko Hidaka, Rich Freiman, and Rolf Sternglanz for the reagents for two-hybrid assays, Masumi Hidaka for human ORC antibodies and Alain Verreault for HAT assay reagents and encouragement. We thank Viola Ellison, Alain Verreault, and Shou Waga for critical reading of the manuscript.

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