**ORC5L, a New Member of the Human Origin Recognition Complex, Is Deleted in Uterine Leiomyomas and Malignant Myeloid Diseases**

(Received for publication, March 9, 1998, and in revised form, July 14, 1998)

David G. Quintana‡§§, Kelly C. Thome‡§§, Zhi-hui Hou‡, Azra H. Ligon‡‡‡‡, Cynthia C. Morton‡‡‡‡, and Anindya Dutta‡‡‡‡

From the ‡Division of Molecular Oncology, Department of Pathology and **Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

A new member of the human origin recognition complex (ORC) was cloned and identified as ORC5L. HsORC5p is a 50-kDa protein whose sequence is 38% identical and 62% similar to ORC5p from Drosophila melanogaster. Two alleles of ORC5 were identified, one with and one without an evolutionarily conserved purine nucleotide binding motif. HsORC5p is precipitated from cell extracts with HsORC2p and HsORC4p, indicating that it is part of the putative human ORC. The bulk of HsORC5p is in an insoluble nuclear fraction, whereas the other known human ORC subunits (HsORC1p, HsORC2p, and HsORC4p) are easily extracted in the nuclear-soluble fractions and in S100 (HsORC1p). In addition, we identified an alternatively spliced mRNA from the same locus (HsORC5T). HsORC5T also formed a complex with HsORC4p but not with HsORC2p, suggesting it may play a regulatory role in the assembly of different ORC subcomplexes. HsORC5, HsORC5T, and HsORC4 transcripts are abundant in spleen, ovary, and prostate in addition to tissues with high levels of DNA replication like testes and colon mucosa, implicating the human ORC proteins in functions besides DNA replication. Finally, the gene for ORC5L is located at chromosome 7, band q22, in the minimal region deleted in 10% of uterine leiomyomas and in 10–20% of acute myeloid leukemias and myelodysplastic syndromes.

Initiation of eukaryotic DNA replication is understood best in the budding yeast Saccharomyces cerevisiae, in which specific initiation sites are recognized by a multi-subunit complex, the origin recognition complex (ORC) (1–5). All six members of ORC identified in yeast are essential for cell viability (3, 6–11).

ORC is bound to DNA throughout the cell cycle (2, 12), and in G1, ORC recruits other proteins involved in initiation of DNA replication.

Yeast ORC utilizes ATP for binding to DNA and has an ATPase activity that is inhibited by binding to the origin of DNA replication (1, 13). Like the DNA replication initiator proteins in Escherichia coli (dnaA) or the simian virus 40 (T antigen), ATP binding and hydrolysis by the eukaryotic initiator protein could be an important regulator of the initiation process.

Three members of a putative human ORC, homologous to yeast ORC1, ORC2, and ORC4, have been identified so far in mammals, specifically in humans and in mice (14–16). A multisubunit complex of proteins containing known ORC subunits has been identified in Drosophila, Xenopus, and humans, although all components have not been demonstrated to be homologs of yeast ORC. In Xenopus, antibody-mediated depletion of ORC inhibits initiation of DNA replication (17–19), whereas in Drosophila, a conditional mutation in the DmORC2 gene demonstrated the involvement of this gene product in initiation of DNA replication (20). Thus, although specific DNA sequences that bind to ORC and initiate DNA replication have not been identified in any species other than S. cerevisiae, the conservation of ORC subunits in higher eukaryotes makes it likely that human ORC will play a similar role in DNA replication.

We report here the identification of a fourth member of human ORC, ORC5L, which is homologous to ScORC5 and DmORC5 (11, 21). Cloning ORC5L moves us closer toward the ultimate goal of reconstituting the entire human ORC in vitro. In addition, the cytogenetic localization of the gene for this subunit corresponds to a region frequently deleted in the most common pelvic tumor in women, uterine leiomyomas or fibroids, and in malignant myeloid diseases. ORC5L is the first gene corresponding to a replication initiation factor consistently deleted in certain human neoplasms. The gene is named ORC5L, whereas the RNA and protein products are called HsORC5 and HsORC5p, respectively, to distinguish them from corresponding products of other species. Unexpectedly, we detected an alternatively spliced mRNA from the ORC5L locus that produced a C-terminally truncated protein that seems to have properties different from full-length HsORC5p.

**EXPERIMENTAL PROCEDURES**

Cloning, Sequencing, RACE, and RT-PCR—In the expressed sequence tag database (dbEST, National Center for Biotechnology Information), the partial sequence of a human cDNA (AA353934) was found showing significant homology to amino acids 9–88 of ORC5p from Drosophila melanogaster. AA353934 represented the 5′ end of a cDNA clone obtained from human Jurkat T cell mRNA. This clone was obtained and found to contain a 1.9-kb cDNA, which corresponds in size to the mRNA detected by Northern blotting. The sequence has been deposited in GenBank (accession number AF049127). After this work was...
completed, another sequence almost identical to ORC5L was reported (22). It differs from our sequence at nucleotide 162, resulting in a change in amino acid 37 in the putative NTP binding motif (we discuss the significance of this polymorphism later).

RACE was performed using a HeLa cell cDNA library (Marathon-Ready; Clontech) in which cDNAs have been cloned ligated to an adaptor sequence used for priming. For amplification of the 5′ ends, polymerase chain reaction was performed using the adaptor primer (AP1) and gene-specific antisense primers.

A cDNA representing the alternatively spliced form of ORC5 was also found in dbEST (AA375988) and sequenced. We refer to this clone, containing a 1.3-kb cDNA, as HsORC5T. The sequence has been deposited at the GenBank™ accession no. AF081459. The full-length mRNA and protein are referred to as HsORC5 or (HsORC5L) and HsORC5p, respectively, whereas the alternatively spliced mRNA and protein are referred to as HsORC5T and HsORC5Tp, respectively.

For RT-PCR, cDNA was prepared by reverse transcription of total RNA from HeLa or B-lymphoid IgH cells using oligo(dT) primer. HsORC5 and HsORC5T-specific sequences were amplified by two rounds of nested PCR, using primers specific to the 5′ primer common to both HsORC5 and HsORC5T and unique 3′ primers (Fig. 2).

Antibodies, Immunoprecipitation, and Immunoblotting—A 1.9-kb BamHI-XhoI fragment from the cDNA encoding the full-length protein was cloned into the same sites of pSET20 clone to express it fused to a His6-epitope tag. A 55-kDa protein was produced in bacteria, purified on a nickel resin column, and used to raise antibodies in rabbits (Co-calcification experiments). Antibodies against human HsORC2p and HsORC5p have been described elsewhere (16).

Recombinant HsORC5p or HsORC5Tp fused with glutathione S-transferase (GST) were expressed in mammalian cells using the pEBG expression plasmid. PCR with Pfu polymerase was used to introduce a SpeI site into the cDNA immediately upstream from the initiator methionine (ACTAGTATG), and for HsORC5 a KpnI site immediately downstream from the stop codon (TGAGGTACC). For HsORC5T a KpnI site from the pBluescript polylinker was utilized. These sites were used to clone HsORC5 and HsORC5T cDNAs into the pEBG vector such that the GST coding region was fused in-frame to the ORC5 coding sequence.

293T cells transiently transfected for 48 h with pEBG, pEBG-HsORC5, or pEBG-HsORC5T were lysed, and the expressed proteins recovered by affinity purification on glutathione agarose beads. Co-precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting according to standard protocols.

*Cell Culture and Fluorescence in Situ Hybridization (FISH)—* Conditions of HeLa cell culture, synchronization, extraction, and Northern blotting were as described previously (16). Northern analysis of tissue samples was performed on a commercially prepared blot (CLONTECH). For protein work, whole cell extracts were prepared in 8 M urea-containing buffer where indicated. Subcellular fractionation was done by hypotonic disruption in a B-Pesel diluent by an aliquot was inspected under the microscope to ensure that cells were disrupted and nuclei were intact. Nuclei were separated by low speed centrifugation, and S100 was obtained by subsequent centrifugation of the low speed supernatant at 100,000 *g*.

**Human ORC5**

**RESULTS**

A Human Protein Homologous to Drosophila and *S. cerevisiae ORC5*—The cDNA insert encodes a predicted protein of 435 amino acids (Fig. 1), with a calculated molecular mass of 50 kDa and a pI of 8.35. We have identified this clone as human replication origin recognition complex ORC5 (ORC5L). The amino acid sequence of HsORC5p has 38% identity (62% similarity) to that of DmORC5p and 24% identity (48% similarity) to that of ScORC5p.

The initiator methionine codon of HsORC5p is preceded by an untranslated leader sequence of 74 nucleotides that does not contain a stop codon in the same reading frame, so theoretically the protein could have a further N-terminal extension; however, alignment of the human protein with DmORC5p and ScORC5p indicates that homology is noted within 10 amino acids from the N termini of these proteins (Fig. 1), suggesting that the cloned human cDNA represents the correct N terminus protein. Also, the size of the protein produced in rabbit reticulocyte lysates by *in vitro* transcription and translation of the cDNA is the same as that of the protein identified by anti-HsORC5p antibodies in HeLa cell extracts and in tissue extracts (see below, Fig. 3). Finally, 5′-RACE using a HeLa cDNA library and gene-specific antisense primers revealed no upstream extensions.

The sequence of HsORC5p encoded by AA375984 does not contain a putative purine nucleotide binding motif present in ORC5p of other species (Fig. 1, amino acids 37–43 of HsORC5p is RXXAXGK instead of GXXGXXG seen in *S. cerevisiae* and *Drosophila*). ScORC5p has been shown to bind ATP (13); however, in another cDNA representing the N-terminal two-thirds of ORC5L in dbEST (AA375988), the corresponding sequence is GXXAXGK. The latter sequence was confirmed by sequencing a RACE product amplified from a HeLa cDNA library. Because GXXAXGK has been reported to be a functional purine nucleotide binding motif, this result suggests that there are at least two alleles of ORC5L differing from each other in a potentially significant nucleotide binding motif.

Unexpectedly, the 3′ end of the cDNA containing AA375988 was different in sequence from that of the HsORC5 cDNA from nucleotide 953 (GenBank™ accession no. AF081459; see also Fig. 1B). The result is the production of an mRNA that encodes a protein with amino acids 1–293 of HsORC5 followed by a unique sequence of 31 amino acids. This putative protein is designated HsORC5Tp. Further analysis suggests that the variant cDNA is the product of alternative splicing.

**Evidence for HsORC5T**—To ensure that HsORC5T was not an artifact produced during cDNA preparation, we did the following experiments. Northern blot analysis of a variety of cell lines with the unique portion of HsORC5T identified a transcript of approximately the same size as detected by a probe containing the unique C-terminal portion of HsORC5 (Fig. 2A). At equivalent levels of exposure, the abundance of the HsORC5T transcript is estimated by densitometric scanning to be about one-tenth that of the HsORC5 transcript.

Because the resolution of our Northern analysis is insufficient to distinguish between the sizes of the 1.9-kb HsORC5 transcript and the 1.3-kb HsORC5T transcript, RT-PCR was employed to identify positively the alternatively spliced HsORC5T transcript (Fig. 2B). Oligo(dT)-primed cDNA was amplified with a pair of external primers that could amplify either HsORC5 (primers A and C) or HsORC5T (primers A and E), and the products were re-amplified with internal primers specific for HsORC5 (primers B and D) or HsORC5T (primers B and F). Amplifications using plasmids containing either Hs-
ORC5 or HsORC5T cDNA as template demonstrated specificity of the primer pairs (Fig. 2, lanes 7–10). The HsORC5T cDNA clone gave a product of 314 base pairs (Fig. 2, lane 7), and the HsORC5 cDNA clone gave a product of 857 base pairs (Fig. 2, lane 10). As seen in Fig. 2C, both HeLa and IgH1 cells have detectable levels of HsORC5T transcript (lanes 2 and 5) in addition to HsORC5 transcript (lanes 3 and 6).

**Antibodies to HsORC5p—** To study HsORC5 protein, we
raised polyclonal rabbit antibodies to the bacterially produced His<sub>6</sub>-tagged HsORC5p. Anti-HsORC5p antibody recognizes a specific 52-kDa band in SF9 insect cell extracts that have been infected with recombinant baculovirus expressing HsORC5 (Fig. 3, lanes 2 and 4) but not in extracts of cells infected with recombinant baculovirus expressing a mock protein (Fig. 3, lanes 1 and 3). When [35S]methionine-labeled (untagged) HsORC5p was produced in rabbit reticulocyte lysates by <i>in vitro</i> transcription and translation, two polypeptides of 52 and 49 kDa were produced (Fig. 3, lane 9). The smaller protein probably was produced by internal translation initiation or by degradation of the larger protein because the anti-HsORC5p antibody specifically immunoprecipitates both polypeptides from reticulocyte lysates, enriching for the higher molecular mass product (not shown).

Immunoblotting of HeLa cell lysates with the anti-HsORC5p antibody specifically identified a 52-kDa polypeptide (Fig. 3, lanes 5 and 6), which was extracted better by 8 M urea than by boiling cells in Laemmli sample buffer but not detected under less stringent salt or detergent conditions. An additional cellular protein of 40 kDa was detected by the anti-HsORC5p antibody and was easily extracted by a buffer containing 0.1% Nonidet P-40 and 150 mM NaCl. In <i>in vitro</i> transcription and translation of the HsORC5T cDNA produced a protein of 40 kDa and a smaller product of 38 kDa, probably by internal initiation (Fig. 3, lane 10). Thus, the 40-kDa cellular protein detected by the anti-HsORC5p antibody could be the HsORC5T protein. However, this issue will need to be confirmed by raising antibodies that specifically recognize the unique portion of HsORC5Tp. Immunoblotting of lysates of a uterine leiomyoma with a normal karyotype (see below) also detected two bands of 52 and 40 kDa (Fig. 3, lanes 7 and 8), corresponding in size to the bands identified in HeLa cell extracts (lane 6) and to the largest <i>in vitro</i> translated products from HsORC5 and HsORC5T (lanes 9 and 10). Identical results were obtained with extracts from Wi38 primary fibroblasts (not shown).

Subcellular Fractionation of ORC Subunits—A complex containing all four known human ORC subunits has not been identified in somatic cell extracts. One possibility is that one or more of the ORC subunits is not easily extracted under conditions expected to maintain inter-subunit interactions, thereby requiring extraction conditions that result in disruption of the hol-ORC. Indeed, when asynchronous HeLa cells were fractionated, HsORC1p, HsORC2p and HsORC4p were found in the nuclear fraction that is extracted in 0.4M NaCl and 0.02% Nonidet P-40. HsORC1p localized also to the S100 fraction. In contrast, HsORC5p remained bound to the nuclear-insoluble pellet (Fig. 4). Nuclear lamins serve as a positive control for the nuclear-insoluble fraction.

HsORC5p Can Associate with HsORC4p and HsORC2p in
**Human ORC5**

Although the anti-HsORC5p antibody immuno-precipitated HsORC5p from rabbit reticulocyte lysates, it could not immunoprecipitate endogenous cellular HsORC5p extracted in 8 M urea. Hence, we examined association of HsORC5p with other known human ORC subunits by an alternative approach. Transient transfection of pEBG-HsORC5Al allowed expression of a 70-kDa GST-HsORC5p fusion protein in 293T cells. Addition of the GST tag and overexpression of the protein also makes possible the recovery of HsORC5p under mild extraction conditions (250 mM NaCl, 0.1% Nonidet P-40). In parallel cultures, GST alone was expressed from the pEBG vector as a negative control. GST-HsORC5p (or GST) was isolated from cell lysates by affinity purification on glutathione-agarose beads and detected by immunoblotting with anti-GST antibody (Fig. 5, top panel, lanes 3 and 4). Immunoblotting of the associated proteins with anti-HsORC2p (Fig. 5, second panel), anti-HsORC4p (Fig. 5, third panel), or anti-HsORC1p antibody (Fig. 5, bottom panel) antibodies revealed that HsORC2p and...
HsORC4p were copurified specifically with GST-HsORC5p but not with GST alone (lane 4 versus lane 3). The 100-kDa HsORC1p was not detected in the GST-HsORC5p precipitate (lane 4).

To examine whether HsORC5Tp had properties similar to HsORC5p, the former was also expressed in mammalian cells as a GST-HsORC5T fusion protein. Consistent with the greater extractability of endogenous HsORC5Tp relative to endogenous HsORC5p, the level of GST-HsORC5Tp extracted from transfected cells was significantly higher than that of GST-HsORC5p when the cells were extracted in 250 mM NaCl, 0.1% Nonidet P-40 lysis buffer. This difference disappeared when extracts were prepared by boiling cells in Laemmli sample buffer (not shown). Pull-down of the GST-fusion proteins on glutathione-agarose beads and immunoblotting with appropriate antibodies indicates that HsORC4p but not HsORC2p associates with GST-HsORC5Tp (Fig. 5, lane 6). Thus, the C-terminal truncation of ORC5 makes the protein both more soluble and abolishes its ability to enter into a functional holo-ORC.

**Expression of HsORC5 along the Cell Cycle and in Different Tissues**—Of the known human replication initiator factors, mRNA levels of HsORC1 and HsCDC6/Cdc18 are highest at the G1-S transition, and their promoters have been shown to be under control of the E2F transcription factor (26, 27). To examine whether HsORC5 mRNA was regulated similarly, Northern blot analysis was done using asynchronous HeLa cells and cells blocked in M and S phases with nocodazole and hydroxyurea, respectively (Fig. 6A). Comparison with the GAPDH control indicates that HsORC5 mRNA essentially is unchanged between M and S phases. HeLa cells also were followed as they progressed synchronously following release from an M phase block (Fig. 6B). Progressive decline and increase of cyclin B mRNA levels indicates that cells passed through G1 and S synchronously. HsORC5 mRNA, however, was detected at a constant level throughout the cell cycle. These results were confirmed in different mRNA preparations and by quantitative comparison of HsORC5 and GAPDH signals by densitometric scanning. Therefore, unlike HsORC1 or HsCDC6, the ORC5L gene is not up-regulated at the G1-S transition. In agreement with the mRNA results, no differences were observed in the level of HsORC5p in whole cell extracts along the cell cycle (not shown).

To examine whether ORC subunit expression varies between tissues, a Northern blot of mRNA from a panel of human tissues was hybridized sequentially with HsORC5-, HsORC5T-, HsORC4-, and HsORC1-specific probes. High levels of HsORC5, HsORC5T, and HsORC4 were observed in testis and prostate (Fig. 6, lanes 3 and 4). HsORC5 and HsORC5T also were expressed at increased levels in spleen and ovary (Fig. 6, lanes 1 and 5). Murine ORC2L expression also has been reported to be highest in testis and high in other non-proliferating tissues such as kidney and brain (15). In contrast, HsORC1 expression was high only in testis and colon mucosal lining (Fig. 6C, lanes 4 and 7). Comparison with the GAPDH loading control suggests that HsORC1 levels are higher in colon mucosal lining than in testis. Thus, whereas HsORC1 expression seems positively correlated to cell proliferation, the expression of the other subunits was higher in tissues without significant proliferation (e.g., spleen, prostate, ovary). In addition, comparison of tissues with similar low levels of proliferation activity (e.g., spleen, thymus, prostate, peripheral blood leukocytes) or tissues with high proliferation activity (small intestine and colon mucosal lining) shows no correlation between proliferation and expression of the HsORC4, HsORC5, and murine ORC2 subunits.

**Cytogenetic Mapping of the ORC5L Gene to 7q22 in a Minimal Region Frequently Deleted in Uterine Leiomyomas and in Malignant Myeloid Disease**—Nucleotides 1224–1889 of Hs-ORC5 cDNA are represented by two exons present in a 162-kb
genomic fragment (bacmid RG126M09) (GenBank accession no. AC002067). Nucleotides 1224–1336 of the cDNA are encoded by an exon composed of nucleotides 13204–13316 of RG126M09, whereas nucleotides 1337–1889 of the cDNA represent an exon containing nucleotides 23204–23756 of the genomic fragment (Fig. 7A). Exons coding for sequences upstream of nucleotide 1224 are not present on this bacmid, indicating the existence of an intron greater than 13 kb. The bacmid also contains the sequence-tagged sites sWSS1097 (also known as D7S658) (nucleotides 63971–64311 of RG126M09) and sWSS1679 (nucleotides 105613–105843 of RG126M09). Therefore, the ORC5L gene is localized to human chromosome 7q22 in an interval that is frequently deleted in uterine leiomyomas and malignant myeloid disease. A, schematic of HsORC5 and HsORC5T cDNAs (bottom), the RG126M09 genomic clone (middle), and the sequence-tagged sites in 7q22 (top) (not to scale). Stippled boxes indicate minimal areas of overlapping deletion mapped in leiomyomas (29–31) and malignant myeloid disease (32, 33). Numbers on the cDNAs and on the RG126M09 genomic clone refer to nucleotide sequence positions. The two sequenced exons of ORC5L present in RG126M09 are indicated. The two sequence-tagged sites in boxes, sWSS1679 and sWSS1097, are present on this same genomic clone at the positions indicated. The localization of ORC5L is indicated by a thick double-headed arrow below the top line; the orientation of the gene is unknown. B, FISH of a metaphase spread from a leiomyoma with del(7)(q22q32). Left, alpha satellite control probe for chromosome 7. The chromosome at the top is a deleted 7q. Right, FISH with ORC5L genomic clone. The arrow marks the absence of ORC5L from the deleted 7q.
7 in band q22, adjacent to and upstream from sWSS1097. These markers fall in contig sWSS6-N (National Human Genome Research Institute data base) or contig WC7.6 (Whitehead Institute radiation hybrid data base), within 3.5 centimorgans (approximately 1 Mb) of markers D7S518, D7S666, and D7S515 (Fig. 7A). The order of markers is centromere-D7S518-D7S666-D7S515-D7S658-D7S501-D7S496-D7S471-telomere. Interest in this region of the genome is high because the interval from D7S518 to D7S471 is the smallest common region of loss of heterozygosity seen in approximately 10% of uterine leiomyomas (28–31).

A smaller interval in this same region (from sWSS3517 to sWSS2526, Fig. 7A) is deleted consistently in 10% of de novo cases of acute myeloid leukemia and myelodysplastic syndrome and in nearly 50% of the therapy-induced cases (32, 33). Leukemias arising in individuals with cancer predisposition syndromes (e.g., Fanconi anemia) usually have loss of heterozygosity in this same region as the only cytogenetic anomaly (32, 33). The latter small region is now cloned as a contig of YACs that encodes about 2 Mb of genomic DNA.

Therefore, mapping of ORC5L to a region within 40 kb of D7S658 places the gene within the smallest interval consistently deleted in a sizable fraction of uterine leiomyomas and malignant myeloid disease.

To confirm this mapping, a 7-kb genomic DNA fragment cloned in lambda gt10 was isolated by hybridization with the HsORC5 cDNA. FISH with this genomic clone on metaphase spreads of normal human lymphocytes confirmed location of the gene to 7q22 (data not shown). Tumor cells from two uterine leiomyomas containing del(7)(q22q32) were hybridized with the ORC5L genomic clone (Fig. 7B, right) and an alpha satellite control probe for chromosome 7 (Fig. 7B, left). In both cases, one copy of the ORC5L gene was deleted in the metaphases examined (ST94–114 is shown in Fig. 7B).

To rule out the possibility that HsORC5T was the product of a different gene, FISH was carried out using multiple probes that contain HsORC5 (both genomic and cDNA) and HsORC5T (cDNA) sequences. Both HsORC5 and HsORC5T signals were detected only at 7q22 (not shown), strongly suggesting that the HsORC5T transcript is produced from the same locus as HsORC5.

**DISCUSSION**

We report here identification of a fourth member of the human replication origin recognition complex, ORC5L, which is homologous to DmORC5 and ScORC5. We also show that HsORC5p can associate with endogenous HsORC2p and HsORC4p in vivo, which argues in favor of the existence of a human origin recognition complex. However, we have not yet detected HsORC1p in these complexes, suggesting that in human somatic cells the multi-subunit ORC may not be as abundant or as stable as in yeast and as in Xenopus egg or Drosophila embryo extracts. Cellular HsORC1p may be present in a stable complex with endogenous ORC subunits and unavailable to form a complex with exogenous epitope-tagged ORC subunits.

The identification of the fourth subunit of the putative human origin recognition complex allowed us to compare the cell-cycle regulation of expression of the different subunits, their relative expression in different tissues, and their behavior upon subcellular fractionation.

Although the HsORC1 mRNA level is regulated by the E2F transcription factor (26), HsORC2 (34), HsORC4 (16), and HsORC5 are expressed constitutively throughout the cell cycle at the mRNA level. HsORC1p, however, is detected at a constant level through the cell cycle in whole cell extracts (34). The constant abundance of all ORC subunits through the cell cycle of human somatic cells is similar to that observed in yeast. Future experiments will be directed toward examining whether some regulation is exerted at the level of ORC assembly or in activation of DNA or protein binding by ORC during specific stages of the cell cycle.

The localization of the different ORC subunits to different subcellular fractions has also been studied. Detection of HsORC1p in the S100 fraction under conditions in which HsORC2p, HsORC4p, and HsORC5p are detected only in the nuclear extracts indicates either of the following possibilities. HsORC1p may be more weakly bound to the chromatin than the other ORC subunits, either through the cell cycle or during some of its phases, thus being released to the cytoplasm during the fractionation process. Indeed, we have reported that when HeLa cell extracts are fractionated over a Bio-Rex 70 column, HsORC1p and HsCDC6/Cdc18p co-elute together but separately from HsORC2p (34) and HsORC4p. Therefore, partial cytoplasmic localization of HsORC1p may be an artifact of the fractionation procedure itself. Many nuclear proteins are quantitatively recovered in the S100 fraction, as is the case for replication factor RPA (35). A different possibility is that HsORC1p is a partner of human HsCDC6/Cdc18p and is subject to similar cell cycle-specific changes in subcellular localization as reported for HsCDC6/Cdc18p (34). Immunofluorescence microscopy experiments currently under way in our laboratory will address this point. In contrast, HsORC5p remains bound to the nuclear-insoluble pellet under conditions extracting all of the other ORC subunits inspected. Also, the finding that HsORC5p is extracted poorly under conditions usually employed to maintain protein-protein interactions might account for the difficulty in identifying the endogenous complete origin recognition complex in human somatic cell extracts. Further research will determine whether the HsORC5p in the nuclear-insoluble fraction is colocalized with replication factories and origins of replication reported to be associated with the nuclear matrix.

The expression of the identified human ORC subunits in different tissues has also been examined. The high levels of HsORC5 and HsORC4 (this paper) and of murine ORC2L (15) transcripts in non-proliferating tissues suggest that human ORC may have functions independent of cell proliferation. ORC has been shown to be involved in silencing in yeast (3, 9, 11). More recently, Pak et al. (36) have shown that Drosophila ORC and the heterochromatin protein HP-1 colocalize to heterochromatin and that DmORC2 is a dosage-sensitive modifier of position effect variegation. At this point, we do not know whether the observed pattern of expression of the different ORC subunits in non-proliferating tissues is related to this or to a different function. On the other hand, HsORC1 is the human ORC subunit that (in addition to high expression in testis) shows the highest level of expression in a mitotically proliferating tissue as the colon mucosal lining. In addition, of the four human ORC members identified to date, HsORC1 is the only one whose transcription is cell cycle regulated. All of this suggests a critical role for HsORC1 in the formation of replication-competent ORC complexes in proliferating human cells. In this regard, we find levels of HsORC2p and HsORC4p unchanged, but HsORC1p levels significantly reduced when WI38 primary fibroblasts are driven into G0 by serum starvation. An intriguing possibility arises from the identification of HsORC5T, an alternatively spliced form of HsORC5. In experiments where HsORC5p interacts both with HsORC2p and HsORC4p, HsORC5Tp interacts only with HsORC4p. This sug-

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2 D. G. Quintana and A. Dutta, unpublished results.
gests a possible regulatory role for HsORC5Tp. HsORC5Tp might assemble a complex containing only a subset of the ORC subunits, unable to fire origins of replication. Experiments are currently under way to determine whether overexpression of HsORC5Tp abolishes replication. Whether such a putative subcomplex would have a function different than replication also deserves further investigation. In addition, the unique C-terminal sequence in HsORC5Tp might recruit alternative partners to the complexes of which it is a subunit. Comparison of proteins pulled down with GST-HsORC5Tp but not with GST-HsORC5p will allow us the identification of specific interactors that might give clues on the role of alternatively spliced HsORC5Tp.

We also report here loss of one copy of ORC5L in uterine leiomyomas and in malignant myeloid diseases. To our knowledge, this is the first description of the frequent loss of one copy of an essential DNA replication gene in human tumors. It is possible that loss of ORC5L may be detrimental for tumor cell growth. Null mutations of the ORC5 gene in Saccharomyces cerevisiae result in non-viability (11, 37). Likewise, immunodepletion of XiORCp (containing XiORC5p) completely stops DNA replication in Xenopus egg extracts (17–19). Although uterine leiomyomas are benign tumors present in at least 20–30% of women over the age of 30, and although they could grow to enormous size, they very rarely, if ever, progress to malignant tumors. Loss of one copy of the gene might diminish levels of HsORC5 mRNA and protein so as to retard the cell cycle and prevent the abundant proliferation and genomic instability necessary for progression to malignancy. Consistent with this possibility, we have reported earlier that (α) myomas in the del(7)(q) subgroup, typically involving deletions of 7q22-q32, are smaller in size than myomas with other cytogenetic lesions (15). Both ORC2 and ORC5 are required for transcriptional silencing, leading to increased expression of oncogenes and growth factors. Decreased HsOrc5p in even a fraction of tumor cells could elevate local levels of secreted growth factors. Decreased HsOrc5p in even a fraction of tumor cells could elevate local levels of secreted growth factors. Decreased HsOrc5p in even a fraction of tumor cells could elevate local levels of secreted growth factors.

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