Association of Human Origin Recognition Complex 1 with Chromatin DNA and Nuclease-resistant Nuclear Structures*

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An origin recognition complex (ORC) consisting of six polypeptides has been identified as a DNA replication origin-binding factor in Saccharomyces cerevisiae. Homologues of ORC subunits have been discovered among eukaryotes, and we have prepared monoclonal antibodies against a human homologue of ORC1 (hORC1) to study its localization in human cells. It was thus found to associate with nuclei throughout the cell cycle and to be resistant to nonionic detergent treatment, in contrast to MCM proteins, which are other replication factors, the association of which with nuclei is clearly dependent on the phase of the cell cycle. A characteristic feature of hORC1 is dissociation by NaCl in a narrow concentration range around 0.25 M, suggesting interaction with some specific partner(s) in nuclei. Nuclease treatment experiments and UV cross-linking experiments further indicated interaction with both nuclease-resistant nuclear structures and chromatin DNA. Although its DNA binding was unaffected, some variation in the cell cycle was apparent, the association with nuclear structures being less stable in the M phase. Interestingly, the less stable association occurred concomitantly with hyperphosphorylation of hORC1, suggesting that this hyperphosphorylation may be involved in M phase changes.

The replication of eukaryotic chromosome occurs in a highly regulated manner during the S phase. It is important to understand coordinated action of various proteins acting on specific cis-elements during initiation of chromosomal replication. In budding yeast (Saccharomyces cerevisiae), the origin recognition complex (ORC),† composed of six polypeptides, is essential for initiation of DNA replication, binding specifically to replication origins throughout the cell cycle (1–6). Prior to the initiation of DNA replication, ORC forms a large protein complex, called a prereplicative complex, by association with other initiation factors, including CDC6 and MCM proteins. After the initiation of DNA replication, the prereplicative complex change to the postreplicative form as indicated by dissociation of factors from ORC in parallel with alteration of the ORC-DNA interaction (3, 5–8). Thus, dynamic change of DNA-protein complexes at replication origins in S phase appears to be closely connected with the initiation of DNA replication.

The counterparts of prereplicative complex components, such as ORC, CDC6, and MCMs, have been identified in various eukaryotes. Thus, the basic mechanisms for initiation of DNA replication seem to be highly conserved. Putative ORC subunits constituting similar multiprotein complexes as that in S. cerevisiae have been identified in Drosophila (9, 10) and Xenopus (11–15). In the latter, depletion of ORC from egg extracts results in inhibition of the initiation of DNA replication (11–14). Similarly, in Drosophila, a conditional mutation in the DmORC2 gene demonstrated strong effects (16). In human cells, putative ORC genes have also been identified (15, 17–23), and interaction of their products has been reported (17, 21–23), suggesting that a similar protein complex might be functional during DNA replication in human cells.

Recent studies have demonstrated that several events in nuclei, including DNA replication, take place in specialized compartments, in which specific factors assemble in highly organized structures. It is essential to ascertain the distribution of replication proteins among subcellular fractions and their interactions to understand how DNA replication is initiated and otherwise regulated. The subcellular localization of MCMs has been well characterized in eukaryotes from yeast to mammalian cells; they associate with prereplicative chromatin and dissociate from it upon initiation of replication (5, 8, 24–27). Subcellular localization of CDC6 proteins also appears to be cell cycle dependent in mammalian cells (28–31). CDC6 is in nuclei in G1 phase and transferred to the cytoplasm when cells enter into S phase. Change in localization of the two factors, MCMs and CDC6, implies a mechanism for prevention of re-initiation of chromosomal DNA. In contrast, ORC in budding yeast associates with chromatin DNA in a sequence-specific manner throughout the cell cycle, suggesting that ORC functions as the landing pad for CDC6, MCMs and other factors to form prereplicative complex (3, 5, 8). The cellular localization of ORC in higher eukaryotes has not yet been elucidated in detail except for the Xenopus case, in which ORC dissociates from metaphase chromatin in an egg extract cell-free system (13, 32).

In mammalian cells, the mRNA expression of ORC1 is cell cycle-regulated by an E2F responsible promoter like that of CDC6 (18). In addition, the abundance of Drosophila ORC1 protein changes with the development stage and the cell cycle, accumulating in proliferating cells in G1/S (33). Furthermore, ectopic expression of ORC1 during Drosophila development alters the proliferation program, suggesting that ORC1 is a main contributor to cell cycle regulation (33). In contrast to ORC1, expression of ORC2–5 seems not be cell cycle regulated in human cells (21, 22, 28, 34). Thus, although ectopic expression of ORC1 in mammalian cells does not drive cells into S phase (18), ORC1 may be one of limiting factor for initiation of

* This work was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan. 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.

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† The abbreviations used are: ORC, origin recognition complex; hORC, human ORC; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter; MCM, minichromosome maintenance.
their replication (34). Elucidation of its precise localization and dynamics in human cells should help uncover roles of the ORC complex in chromosomal DNA replication. This was the aim of the present cell fractionation and immunostaining study with a newly prepared anti-human ORC1 monoclonal antibody.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Synchronization**—HeLa S3 and normal human fibroblast WI38 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS). For synchronization, exponentially growing HeLa S3 cells were arrested at M phase for 24 h with 150 ng/ml of TN16 (WAKO, Osaka, Japan) or with 2.5 mM of thymidine at partially growing HeLa S3 cells were arrested at M phase for 24 h with 150 ng/ml TN16 (WAKO, Osaka, Japan) or with 2.5 mM of thymidine (amino acids 1–698) tagged with His6 at the N terminus (His hORC1N) and anti-hORC1 protein, appearing occasionally at various levels. Clones were screened by using human homologue on immunoblotting (25). Goat anti-lamin B antibody was purchased from Santa Cruz Biotechnology. Antibodies—Hybridoma cell lines were prepared from Balb/c mice after immunization with synthetic 15-amino acid polypeptides from within the human ORC amino acid sequence. Clones were screened by their reactivity against a recombinant human ORC1 (hORC1) protein (amino acids 1–698) tagged with His6 at the N terminus (His hORC1N) by immunoblotting. Two positive clones, 3C1B (producing IgG1) and 3A2A (producing IgG1) were obtained and used in this study. Rabbit anti-murine MCM3 (mMCM3) antisera was kindly provided by Dr. H. Kimura (Oxford University). This antibody recognized the corresponding human homologue on immunoblotting (25). Goat anti-lamin B antibody was purchased from Santa Cruz Biotechnology.

**Immunoprecipitation and Phosphatase Treatment**—For immunoprecipitation of hORC1, anti-hORC1 antibody (3A2A) was added to 0.2 M NaCl extracts of HeLa S3 cells as described above at a concentration of 20 μg/ml. After 8 h incubation at 4 °C, protein A-Sepharose (Amer- sham Pharmacia Biotech) was added and incubated for an additional 2 h. After centrifugation, the supernatant (sup) and precipitated (ppt) fractions were analyzed by immunoblotting with 3A2A.

4 °C, the debris was washed once more with the same volume of ice-cold 0.1% TX-100mCSK. For DNase I or NaCl extraction, the debris from the second 0.1% TX-100mCSK extraction was resuspended in 1 ml of ice-cold 0.1% TX-100mCSK supplemented with indicated amounts of NaCl or DNase I (Roche Molecular Biochemicals) and incubated further at the indicated temperature. After incubation, insoluble and soluble materials were separated by centrifugation. The chromatin DNA re- tained after this DNase I treatment was isolated from the insoluble fraction by treatment with 20 μg/ml EDTA, 0.5% SDS, and 100 μg/ml RNase A in 0.1% TX-100mCSK at 37 °C for 30 min followed by 2 h incubation with Protease K (200 μg/ml). The DNA was extracted with phenol-chloroform (1:1), precipitated with ethanol, separated by electrophoresis in 0.8% agarose, and quantified with an image analyzer (ATTO, Tokyo, Japan).

**Fig. 1. Detection of cellular hORC1 protein with a monoclonal antibody.** A, 100 ng of purified His-hORC1N and total cell lysate from 1 × 10⁶ HeLa S3 cells (HeLa S3 Total) were subjected to immunoblotting with 3A2A. The filled and open arrowheads indicate hORC1 and His-hORC1N, respectively. The weak additional band positioned at ~60 kDa in HeLa S3 total cell lysate is a degradation product of hORC1. B, hORC1 was immuno- purified from a 0.2 M NaCl extract of HeLa S3 cells with 3A2A. Total cell lysate obtained from asynchronous cells before incubation with 3A2A (upper panel) and after centrifugation and detection with 3A2A (upper panel). Immunoprecipitates from the indicated numbers of cells (lanes 7–9) were subjected to SDS-PAGE and stained with Coomassie Blue (lower panel). In lane 10, Rainbow-colored protein molecular weight markers (Amersham Pharmacia Biotech) were run as a size marker. The filled arrowhead indicates hORC1, and open arrowheads indicate IgG (top and bottom arrow indicates heavy and light chains, respectively).

**Fig. 2. Nuclear hORC1 protein associated with nonionic detergent-resistant nuclear structures.** A, the cytoplasmic fraction (S100), the 0.2 M NaCl-soluble fraction (NE), and the remaining insoluble fraction (ppt) from 5 × 10⁶ kidney 293 cells were immunoblotted with 3A2A. B, HeLa S3 cells, growing asynchronously (Asyn) (lanes 2 and 3) or arrested with TN16 at M phase (lanes 4 and 5) were treated with 0.1% TX-100mCSK, and the supernatant (sup) (lanes 2 and 4) and the precipitated fractions were obtained after centrifugation (ppt) (lanes 3 and 5). Proteins from 2 × 10⁶ cells were analyzed by immunoblotting with 3A2A. The total cell lysate obtained from asynchronous cells before fractionation is shown in lane 1. FACS analyses of the used cell preparations are shown in the top panels. C, the insoluble fraction from HeLa S3 cells after Triton extraction was incubated on ice for 30 min in 0.1% TX-CSK supplemented with the indicated concentrations of NaCl. After centrifugation, the supernatant (sup) and precipitated (ppt) fractions were analyzed by immunoblotting with 3A2A.
To test the shift of hORC1 by phosphorylation, the immunoprecipitate was treated with λ phosphatase (20 units/μl; New England Biolabs) in 1 X λ phosphatase buffer (50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 2 mM MnCl2, 5 mM dithiothreitol, 0.01% Brij 35, 0.1 mg/ml bovine serum albumin) at 30 °C for 30 min. The reaction was stopped by the addition of SDS sample buffer, and the products were separated by SDS-PAGE.

Western Blotting—All procedures were performed at room temperature except for blotting at 4 °C. Samples were separated by 12.5% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Amersham Pharmacia Biotech) using a semidy transfer apparatus in transfer solution (100 mM Tris, 192 mM glycine, 0.1% SDS, pH 6.3) at 3 mA/cm2 for 30 min. After blocking of membranes, achieved with Tris-buffered saline + 0.1% Tween 20 containing 10% dry milk (SNOW Brand, Sapporo, Japan) at 4 °C overnight, they were incubated first with antibodies in Tris-buffered saline + 0.1% Tween 20 containing 20% FCS for 1 h, followed by peroxidase-labeled second antibodies in the same solution for 1 h. Antibody reactive protein bands were visualized using the ECL system (Amersham Pharmacia Biotech).

Immunostaining—All procedures were carried out at room temperature. Cells on cover glasses were washed with CSK buffer without Triton three times, and fixed with 2.5% formalin solution in CSK for 10 min. After three washes with CSK, the fixed cells were incubated with PBS containing 10% FCS and 0.1% Triton for 30 min, for masking and permeabilization. Following a further three washes with 10% FCS in PBS, the cover glasses were incubated with the culture supernatant of clone 3C1B for 1 h, washed with 10% FCS in PBS three times, and incubated with Rhodamin-conjugated second antibody (MBL, Nagoya, Japan; reactive with both IgM and IgG) and 0.02 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) in PBS containing 10% FCS for 1 h. After washing with PBS four times, the samples were mounted on slide glasses, and fluorescent signals were visualized with a Leica DMRBE and captured with a Photometrics PXL.

UV Cross-linking—HeLa S3 cells grown in 100-mm dishes (5 X 106 cells) were washed twice with PBS and irradiated with UV at the indicated doses in an UV chamber (Spectronics Corp., Westbury, NY) in 10 ml of PBS. The cells were lysed in 500 μl of 0.1% TX-100mCSK at 4 °C for 10 min, and the insoluble fraction was obtained after centrifugation. NaCl extracts were prepared as described under “Fractionations of Cellular Proteins,” above.

RESULTS

Detection of hORC1 from Human Cell Lysates—We have developed hybridoma cell lines expressing antibodies 3A2A and 3C1B against specific oligopeptides of the hORC1 protein. Because they were screened for a recombinant hORC1 fragment produced in Escherichia coli (containing amino acids 1–698), it was necessary to test whether they could detect hORC1 protein specifically in human cell lysates. When we applied 1 X 105 HeLa cells to an immunoblot analysis, a major protein band of 97 kDa could be detected with monoclonal antibodies 3A2A (Fig. 1A) and 3C1B (data not shown). As the molecular mass of the detected band was in good agreement with the predicted molecular mass of hORC1, it was concluded that these antibodies specifically recognize hORC1 protein.

Next we estimated the amount of hORC1 in human cells by the immuno purification method. First, the recovery of hORC1 by immunoprecipitation was calculated as follows. Cellular proteins from a fixed number of HeLa cells were prepared by using: 1) a total cell lysate obtained by SDS lysis, and 2) a soluble fraction obtained as an 0.2 M NaCl extract. About 50% of hORC1 in the total cell lysate was recovered in the latter soluble fraction (Fig. 1B, upper panel). Because about 80% of hORC1 in the 0.2 M NaCl extract was recovered in the immunoprecipitated fraction (Fig. 1B, upper panel), 3A2A could precipitate the native hORC1 protein in the cell extract efficiently. Thus, 40% of the cellular hORC1 protein could be recovered by immunoprecipitation. When the immunoprecipitate from HeLa cells was analyzed by SDS-PAGE followed by Coomassie Blue staining, only a hORC1 band appeared other than the heavy and light chains of the IgG (Fig. 1B, lower panel), indicating...
that hORC1 in the 0.2 mM NaCl extract is mostly a single molecule entity. Using bovine serum albumin bands as the standard, we estimated that about 1 μg of hORC1 was recovered from 1 × 10^7 cells (Fig. 1B, lower panel). Using the above factors for extraction and immunoprecipitation efficiencies, the cellular content of hORC1 was calculated to be approximately 1.6 × 10^9 molecules per HeLa cell.

**Association of hORC1 with Nonionic Detergent-resistant Nuclear Structure throughout Cell Cycle**—The subcellular localization of hORC1 was roughly studied with a cytoplasmic extract, 0.2 mM NaCl nuclear extract, and the remaining cell debris from 293 cells. As shown in Fig. 2A, the majority of hORC1 was detected in the 0.2 mM extract, suggesting a predominant localization in nuclei. It is known that nuclear proteins associated with some nuclear structures are retained in nuclei even after nonionic detergent treatment. When HeLa cells growing synchronously and arrested in M phase by TN16 were treated with CSK buffer containing 0.1% Triton (0.1% TX-100 mCSK), all hORC1 remained the insoluble fraction (Fig. 2B). In contrast the other replication factors, MCMs were separated in two forms, soluble and insoluble, by this treatment (see Fig. 5A). It is noteworthy that a mobility shift of hORC1 in SDS-PAGE was observed in M phase-arrested cells, suggesting some modification as described below.

Solubility of hORC1 in the Triton-insoluble fraction was studied with increasing concentrations of NaCl, as shown in Fig. 2C. Interestingly, hORC1 was completely insoluble with 100 mM NaCl, whereas more than 90% of hORC1 became soluble with 250 mM NaCl.

To obtain convincing evidence of nuclear localization of hORC1 by fractionation of cellular proteins, immunostaining of HeLa cells was performed with the 3C1B antibody. As shown in Fig. 3, hORC1 was found in the nuclei in both unwashed (Fig. 3A) and Triton-washed HeLa cells (Fig. 3B). However, after extraction with 250 mM NaCl, hORC1 signals could no longer be detected (Fig. 3C), consistent with the cell fractionation experiments, indicating that hORC1 mainly exists in nuclei, but it efficiently dissociated at 0.25 mM NaCl.

To investigate the localization of hORC1 during the cell cycle, synchronously growing WI38 cells were studied (Fig. 4). Flow cytometric analysis revealed more than 90% of cells to be arrested once in G$_0$ and to enter S phase at 20 or 24 h after serum addition (Fig. 4A). The total cell lysate was subjected to SDS-PAGE (Fig. 4B), and hORC1 and MCM3 were detected by immunoblotting (Fig. 4C). These results demonstrated hORC1 protein to begin to increase at 12–16 h, just before the onset of S phase, as also observed for MCM3 proteins. Using the same samples, retention of hORC1 in nuclei was tested by detergent treatment. As shown in Fig. 4D, all hORC1 existed in the insoluble fraction throughout the cell cycle except for partial release in the 16 h sample. Because the increase of hORC1 protein was greatest during 12–16 h, the soluble protein may correspond to newly synthesized molecules not yet imported into the nuclei.

**Association of hORC1 with Both Chromatin DNA and Nucleosome-resistant Nuclear Structure**—In line with a previous report (36), about half of the MCM3 in cells became soluble with Triton, but the rest remained insoluble (Fig. 5A, lanes 2 and 3). Furthermore, the latter was released from nuclear debris, in parallel with solubilization of DNA by DNase I treatment, indicating a chromatin-bound nature (Ref. 36; Fig. 5, A, lanes 4–11, and B). Association of MCMs with chromatin DNA was also demonstrated by UV cross-linking experiments, as shown in Fig. 5, C and D. The chromatin-bound MCM3, soluble on 0.5 mM NaCl treatment (Ref. 36; data not shown), became insoluble upon UV irradiation in a dose dependent manner (Fig. 5, C, lanes 6–10, and D). The dose required to make half of the MCM3 insoluble was calculated to be 100 μJ/cm$^2$. On the other hand, the MCM3 in the Triton-soluble fraction was scarcely cross-linked in this UV range (Fig. 5, C, lanes 1–5, and D). All hORC1 was soluble in 0.5 mM NaCl before UV irradiation (Fig. 2C), becoming insoluble in a UV dose-dependent manner even in the presence of 0.5 mM NaCl, like the Triton-insoluble MCM3 (Fig. 5, C and D). It has been reported that only proteins that associate directly with DNA could be cross-linked by UV (37). Thus, these results suggest that most MCM3 and the hORC1 retained in nuclei after Triton washing are linked directly with chromatin DNA.

To determine whether the same mode of association is involved, the susceptibility of hORC1 to nucleosome treatment in the Triton-insoluble fraction was compared with that of MCM3. On incubation with DNase I for 40 min, when all MCM3 became soluble and less than 20% of total chromatin DNA shorter
than 500 base pairs remained in nuclei (Fig. 5B), none of the hORC1 protein was eluted (Fig. 5A, lanes 10 and 11). The insoluble nature of hORC1 with nuclease treatment points to a link with nuclear structures other than chromatin.

**Phosphorylation of hORC1 in M Phase**—When hORC1 was isolated from M phase-arrested HeLa cells, its migration in SDS-PAGE was slower than that isolated from asynchronously growing cells (Fig. 2B). This change did not occur during the cell lysis process, because the same mobility shift could be detected in the lysate from chloroacetic acid-fixed cells, in which the activity of enzyme is minimized by acid denaturation (Fig. 6A, lane 4). This experiment led to the conclusion that the modification is M phase-specific, because no shift of hORC1 could be detected in asynchronous or thymidine-blocked cells (Fig. 6A, lanes 1–3). To examine the relationship to phosphorylation, hORC1 was purified from TN16-arrested cells by immunoprecipitation, and incubated with λ phosphatase. As shown in Fig. 6B, its mobility shifted down to the position with asynchronous cells (lane 3 and 4). The band shift of hORC1 was not detectable at any time point after the release from double thymidine blocking except for at 9 h, when about a half of the cells were in late M and the remainder were in G1 (Fig. 6C; FACS). At this time, a significant portion of the hORC1 band shifted upward as in the TN16-arrested sample (Fig. 6C, lanes 3 and 6).

**hORC1 in M Phase**—hORC1 was extracted efficiently by around 0.25 M NaCl from the insoluble nuclear fraction, suggesting that some DNA-hORC1 and/or protein-hORC1 interaction is sensitive to this moderate salt condition (Fig. 2C). To study this characteristic, Triton-washed nuclei were prepared from asynchronously growing cells, as well as in S phase by thymidine blocking or in M phase by TN16 treatment and further treated with increasing concentrations of NaCl (Fig. 7A). The salt sensitivity of hORC1 retention in S phase was almost same as that from asynchronous cells, and most hORC1 protein was eluted at 250 mM NaCl (Fig. 7A, Asyn and S phase arrest). The retention became sensitive to salt in M phase, and most hORC1 eluted at 200 mM (Fig. 7A, M phase arrest). Nuclear lamin B serving as a control was resistant to salt in asynchronous cells, but soluble only by Triton-washing in M phase (Fig. 7A, Lamin B). These results suggested that the association of hORC1 with chromatin DNA or nuclease-resistant structures becomes weak in M phase. As shown in Fig. 7B, hORC1 in M phase was cross-linked with chromatin as efficiently as in asynchronous cells. On the other hand, half of the hORC1 in M phase was eluted by DNase I treatment (Fig. 7C). Under these conditions, the efficiency of solubilization of chromatin DNA was similar to that in asynchronous cells (data not shown). To eliminate the possibility that the alteration of hORC1 association with nuclear structure is due to the effect of the M phase arrest by microtubule disrupting drug, we tested its elution by DNase I treatment with M phase-enriched cells from double thymidine block synchronization. In cells at 9 h after the release from double thymidine block, a significant amount of hORC1 was shifted upward by phosphorylation and partly eluted by DNase I treatment (Fig. 7D, 9 h), whereas the shift and elution by DNase I were limited at the other time points (Fig. 7D, 0, 8, 10, and 11 h). It should be noted that two distinct shifted bands appeared in the eluted sample by DNase I: the higher one is at the same position as observed in TN16-arrested cells, and another is at slightly lower position, suggesting multiple phosphorylation of hORC1 during M phase. These results suggest that the association of hORC1 with chromatin DNA in M phase was not affected significantly, whereas that with nuclease-resistant structures was weakened.

**DISCUSSION**

The present analyses of the localization of hORC1 protein in human cells, using newly prepared hORC1-specific monoclonal antibodies, provided evidence of a direct link with chromatin...
concentrations of NaCl (100, 150, 200, and 250 mM) as described for Fig. 4. Asyn, asynchronous cell, arrested with TN16 at M phase, and arrested DNA and a nuclease-resistant nuclear component(s) is possible. Alternatively, interaction with both chromatin partners. Taking the factors necessary to release hORC1 from wash within a narrow concentration band pointing to limited resistance to nuclease, it was easily eluted by a simple salt Triton-insoluble fraction by DNase I treatment, suggesting DNA. However, unlike MCM, hORC1 was not eluted from the Triton-insoluble fraction by DNase I treatment, pointing to a common origin of ORC binding site promptly after the loading. Indeed, a previous report indicated that chromatin-bound MCMs are separated from hORC2 by at least 500–1000 base pairs in HeLa cell chromosomes (38).

The present study showed the phosphorylated form of hORC1 to appear in M phase-arrested cells and in HeLa cells synchronously progressing from M to G1 phase as a slow migrating band on SDS-PAGE. However, we could not detect the mobility shift in neither asynchronous HeLa cells or in semisynchronously growing normal fibroblasts, so that the slow migrating form of hORC1 appears in only a limited window in the cell cycle, around metaphase. Similar slow migration of XORC1 and XORC2 has been shown in cytostatic factor-arrested extracts of Xenopus eggs (15), pointing to a common modification of ORC1 in M phase. Although the significance of this phosphorylation is unknown, the timing is consistent with that of a potential change of hORC1 association with the nuclear structure. Thus, the link could be causative. Alternatively, the target structure may disappear in M phase or be masked from hORC1 in condensed chromatin. Further experiments are needed to clarify this point.

Although budding yeast ORC has sequence-specific DNA binding activity, none of the ORCs in higher eukaryotes has so far been shown to have such specificity. In contrast to yeast autonomously replicating sequence, mammalian chromosomes can not be expected to have discrete origin sequences (41, 42). However, several pieces of evidence indicate that there are preferential sequences for origins through interaction with specific proteins in somatic cells (43, 44). The present study showed that hORC1 is associated with particular DNA regions that are resistant to nuclease treatment, this being maintained throughout the cell cycle, as observed for DNA binding of yeast ORC (3, 5, 6, 8). If human ORC binds to DNA sequences for initiation of DNA replication through hORC1-DNA interactions, our antibody will be a useful tool to isolate cis-elements from human cells.

In addition to functioning as a replication factor, ORC1 is possibly involved in gene silencing from yeast to higher DNA. It has been reported that ORC behaves as a stable complex in S. cerevisiae, Xenopus egg extract, and Drosophila embryo (9, 10, 12, 14, 15). On the other hand, previous reports have suggested that ORC1 subunit behaves differently from other subunits in human cells, indicating an unstable complex formation of human ORC (22, 28, 34). We showed here that hORC1 is eluted from nuclei by simple salt wash, despite its association with nuclear structures. This is apparently different from tight association of hORC5 with the nuclear-insoluble fraction, being unable to be dissociated even with 0.4 M NaCl (22). We further showed that entire population of ORC1 in human cells have an association with nuclease-resistant nuclear structures throughout the cell cycle except for M phase, demonstrating an obvious contrast with the association of hORC2 with a nuclear fraction, which was highly sensitive to nuclease treatment (38). These results support the previous notion that ORC in human cells would not be in a tight complex, and if they form a complex, their assembly would occur in a narrow time window during cell cycle (34). Indeed our observation indicated that predominant hORC1 in 0.2 M NaCl extract is single molecule entity.

Interestingly, unlike MCMs but similar to hORC1, chromatin-bound CDC6 associates with nuclear structures (29, 30). Because hORC1 physically interacts with hCDC6 in cell extracts (28), it may recruit hCDC6, thereby functioning as a MCM loader as suggested in yeast (39, 40). Although ORCs may determine all sites of MCM loading, the status of their associations with nuclear structures obviously differs. The available data suggest that MCMs may move away from the ORC binding site promptly after the loading. Indeed, a previous report indicated that chromatin-bound MCMs are separated from hORC2 by at least 500–1000 base pairs in HeLa cell chromosomes (38).

Fig. 7. hORC1 in M phase cells. A. cells exponentially growing (Asyn, asynchronous cells), arrested with TN16 at M phase, and arrested with a thymidine block in S phase were treated with the indicated concentrations of NaCl (100, 150, 200, and 250 mM) as described for Fig. 2C and analyzed by immunoblotting with 3A2A and anti-lamin B antibodies. B. UV cross-linking analysis as described for Fig. 5 was performed on TN16-arrested M phase cells. hORC1 and MCM3 were detected by immunoblotting. All MCM3 was extracted by Triton treatment in M phase. C, asynchronous or TN16-arrested cells were incubated with DNase I (DNase +) or without (DNase −) for 40 min as described for Fig. 5. After centrifugation, hORC1 in soluble (sup) and insoluble (ppt) fractions was analyzed by immunoblotting. D, HeLa S3 cells were released from double thymidine blocking and harvested at the indicated time points. The Triton-insoluble fractions from these cells as shown in the left lane (Triton ppt) were treated with (+) or without (−) DNase I as described for C. Cell populations in samples determined by FACS analyses are shown the right.
eukaryotes. In yeast, ORC1 directly associates with SIR1 protein (45), a silencing factor, and mutant alleles for ORC subunits affect gene silencing at mating type and telomeric loci (2, 46, 47). In Drosophila, ORC colocalizes with HP1, a structural component of heterochromatin, and ORC1 physiologically interacts with it (48). In connection with the proposed conserved role of ORC in gene silencing, the features of hORC1 observed in this study are compatible with formation of silencing-related heterochromatin. First, the DNA binding property of hORC1 satisfies the function of tethering heterochromatin components to particular DNA sequences. Indeed, the associated DNA seems to be resistant to nuclease treatment, indicating localization inside of silenced chromatin. Second, its nuclear structure association may reflect a relation with heterochromatin components integrated in insoluble nuclear structures. In this context, it is of interest that HP1 is reported to colocalize with lamin B receptor in human cells (50), which form such actin-related protein (Arp4) in Drosophila (49) and interact with lamin B receptor in human cells (50), which form such nuclear structures.

The available data indicate that hORC1 behaves as both a static and a dynamic component in nuclei and has a key role in controlling cell programs through DNA replication and chromatin organization. To approach the mechanisms, elucidation of functional connections of all ORC subunits on nuclear structures is indispensable. The present study was one step toward this goal.

Acknowledgments—We thank Dr. M. Fujita (Aichi Cancer Center) for helpful and shared discussions, Dr. K. Ohtani (Tokyo Medical and Dental University) for providing hORC1 cDNA, Dr. H. Kimura (Oxford University) for providing anti-mMCM antibody, Dr. K. Takeuchi (NAIST) for helpful advice on establishment of monoclonal antibody, Dr. H. Masumoto (Nagoya University) for advice on preparation of M phase cells, S. Yamaguchi (NAIST) for construction of a plasmid for transfection, Dr. H. Masumoto (Nagoya University) for advice on preparation of M phase cells, S. Yamaguchi (NAIST) for construction of a plasmid for transfection, and Dr. S. Ohta (NAIST) for preparing nuclear extracts from 293S cells.

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*J. Biol. Chem.* 2000, 275:5904-5910.
doi: 10.1074/jbc.275.8.5904

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