Nuclear organization of DNA replication initiation proteins in mammalian cells*

Masatoshi Fujita‡§, Yukio Ishimi¶, Hiromu Nakamura‡, Tohru Kiyono‡
and Tatsuya Tsurumi‡

From the ‡Laboratory of Viral Oncology, Division of Virology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681 and the ¶Mitsubishi Kasei Institute of Life Science, 11 Minamiooya, Machida, Tokyo 194-8511, Japan.

§ Corresponding author:
Masatoshi Fujita
Tel & FAX: 81-52-764-2981.
e-mail:mfujita@aichi-cc.jp

Running title: Organization of human initiation proteins

*This work was supported in part by Grants from the Ministry of Education, Science, Sports and Culture of Japan, and from the Uehara Memorial Foundation.
SUMMARY

ORC, CDC6 and MCM proteins assemble sequentially to form prereplication chromatin. However, their organization remains largely unclear in mammalian cells. Here, we show that ORC1 proteins are associated with non-chromatin nuclear structures and assemble in nuclear foci in mammalian cells using an in vivo chemical cross-linking method. CDC6 proteins were also found to assemble in nuclear foci on non-chromatin nuclear structures although their physical association with ORC1 has been undetectable. In contrast to the situation in yeast cells, CDC6 was found to remain associated with non-chromatin nuclear structures even after cells entered into S phase. Instead, ORC1 proteins were found to be degraded during S phase dependent on proteasomes. We also found that some ORC2 proteins are associated with non-chromatin nuclear structures like ORC1 although the remainder binds to nuclease-sensitive chromatin. Further analyses indicate that ORC2 physically interacts with ORC1 on non-chromatin nuclear structures. On the other hand, our results suggest that although a small proportion of MCM complexes are loaded onto chromatin regions near ORC foci, most of them are more widely distributed. Possible relations between such organization of prereplication chromatin and complicated origin specification in higher eukaryotic cells are discussed.
INTRODUCTION

It has been suggested that nucleic acid metabolism is carried out on spatially organized domain structures in the cell nucleus (reviewed in Ref.1). Non-chromatin nuclear structures such as “the nuclear matrix”, “the scaffold”, and “the nucleoskeleton” has been suggested as key players in organizing high-order chromatin/nuclear structures (reviewed in Refs.2 and 3). In the case of DNA replication, for example, fluorescence microscopic analyses have revealed discrete granular sites of replication, i.e. replication sites or replication foci (4-7). Replication foci may be constructed based on non-chromatin nuclear structures, since nascent DNA and many proteins involved in DNA synthesis have been found to attach to these (8, 9: also reviewed in Refs.2 and 3). In addition, non-chromatin nuclear elements have also been implicated in the initiation step of DNA replication (reviewed in Ref.10); replication origins are often situated close to matrix/scaffold-attachment regions (11-14). In this regard, it is of interest to ascertain how replication initiation proteins such as the origin recognition complex (ORC), CDC6 and MCM, interact with non-chromatin nuclear structures.

The ORC, consisting of six subunits ORC1 to 6, was originally identified as binding specifically to definitive origin sequences in budding yeast (15). Subsequent studies with yeast and Xenopus egg extracts suggested stepwise assembly of ORC, CDC6, and MCM, a six-member protein complex, onto chromatin to form prereplication complexes (reviewed in Refs.16 and 17). Additional protein(s) is
also required for the prereplication complex formation (18, 19). However, the mode of organization remains largely unknown, especially in higher eukaryotes. For example, in the Xenopus egg extract system, it is unknown how ORC interacts with DNA, and so far physical interactions have not been detected among ORC, CDC6, and the MCM complex (20, 21). In addition, in mammalian cells, it is also controversial whether a stable ORC hexamer is present. To understand regulation of DNA replication in mammalian cells, we have been analyzing the spatiotemporal organization of ORC, CDC6 and MCM proteins. We previously developed a method which enables us to remove about 70% of chromatin from HeLa cell nuclei by DNase I digestion under relatively physiological conditions. Using this method, it has been shown that human MCM heterohexamers are associated the removable chromatin while ORC1 and CDC6 proteins bind to nuclease-resistant structures (22-24). As described above, it would be intriguing if ORC1 and CDC6 were associated with nonchromatin nuclear structures. However, this has hitherto not been addressed adequately (for details see below). We here used a method in which viable HeLa cells are first cross-linked with the cell-permeable protein-protein cross-linker dithiobis-succinimidylpropionate (DSP; Ref.25) and then chromatin is extensively stripped. We thereby could show an association of ORC1 and CDC6 protein with non-chromatin nuclear structures in viable cells. We have further extended analyses to dissect the relative organization in nuclei of ORC1, ORC2, CDC6 and MCM proteins.
EXPERIMENTAL PROCEDURES

Cell culture and synchronization
HeLa cells were grown in D-MEM with 8% fetal calf serum and synchronized in early S phase with 2.5mM hydroxyurea or 15µM aphidicolin treatment for 14-18 h, or in G2/M phase with 50ng nocodazole treatment for 14-18 h. Cells in mid to late G1 phase were obtained, as described previously (23). MG132 (Z-Leu-Leu-Leu-CHO) dissolved in DMSO at 20mM was added to cultures at a final concentration of 20µM. As a control, cells treated with 0.1% DMSO were also prepared.

In vivo cross-linking of viable cells with DSP
HeLa cells in 100mm plates were washed three times with phosphate-buffered saline (PBS) at room temperature, mixed with 10ml cross-link buffer (23) and DSP dissolved in DMSO was added to a final concentration of 200µg/ml. The plates were gently swirled for 10min at room temperature, and the reaction was quenched by adding 10ml of TE buffer (10mM Tris-HCl, pH7.6, 1mM EDTA). The cells were then further washed twice with ice-cold PBS.

Cell fractionation
DSP-treated cells were lysed for 5 min on ice with 1ml of ice-cold 0.1%TX-100mCSK buffer (22) without dithiothreitol and ATP. Multiple protease inhibitors, 200µM Na3VO4 and 20mM NaF were also added to the buffer. The cells were then subjected to centrifugation to obtain Triton X-100-extractable fractions. The extracted nuclei
were further extracted with 0.5M NaCl/NP-40 buffer (0.5M NaCl, 1% Nonidet P-40, 10mM Tris-HCl, pH 7.6) and centrifuged to obtain the salt-extractable fractions. The Triton- and salt-extracted nuclei were digested with 1000U/ml DNase I (10U/µl, Boehringer Mannheim) in 0.1%TX-100mCSK containing 2mM MgCl₂ and the multiple protease inhibitors at 25°C for 30min, and then NaCl was added to a final concentration of 0.5M. The samples were then centrifuged to obtain the solubilized chromatin fraction and the remaining non-chromatin nuclear structure. The samples were added to the same volumes of 2x SDS-sample buffer (26) and boiled.

**Antibodies**

A thioredoxin fusion protein containing amino acids 1 to 145 of human ORC2 was produced in *E. coli* according to the manufacturer’s instruction (Invitrogen). Rabbits were injected with the purified fusion protein, and anti-ORC2 antibodies were affinity-purified from the antiserum, as described (23, 26). With Western blotting, anti-ORC2 antibodies recognized a protein migrating at approximately 70kDa in whole cell lysates from HeLa cells, the mobility of which on SDS-PAGE was as expected for ORC2. The antibodies also could immunoprecipitate the 70kDa protein. Together, we concluded that the 70kDa protein is ORC2 (data not shown).

Preparation and specificity of the anti-human MCM7 and CDC6 rabbit polyclonal antibodies were described previously (23, 26). Anti-HA rat (3F10) or mouse (HA.11) monoclonal antibodies were
purchased from Roche and BAbCO, respectively, and an anti-PCNA mouse monoclonal antibody (PC10) from DAKO.

**Immunoprecipitation and immunoblotting**

For immunoprecipitation after in vivo DSP cross-linking, HeLa cells in 100mm plates were lysed on ice in 0.1%TX-100mCSK buffer. The soluble fraction was separated by centrifugation, and SDS was added to a final concentration of 0.15%. The nuclear pellet was resuspended into 100µl of RIPA buffer (26) containing 1.5% SDS, diluted with NET gel buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 0.1% Triton X-100, 1mM EDTA), and sonicated. Separate aliquots of the lysates were immunoprecipitated with antibody-fixed beads.

For immunoprecipitation with anti-HA antibody, 0.1%TX-100mCSK-extracted nuclei isolated as described above were resuspended into 1ml of 250NTT buffer (250mM NaCl, 0.5% Triton X-100, 50mM Tris-HCl pH7.5), and the nuclear extracts were clarified by centrifugation. Separate aliquots of the extracts were then mixed with the anti-HA antibody 3F10 and further incubated after addition of protein G-agarose beads. The beads were washed with 250NTT buffer.

Immunoblotting was performed as described previously (26). Antibody binding was visualized using the ECL system (Amersham).

**Immunofluorescence analyses**

All staining procedures were carried out at room temperature except for extraction and incubation with primary antibodies. Cells grown
in culture chambers (Nunc) were washed with PBS, fixed for 10 min with 3.7% formaldehyde, and then permeabilized with methanol for 2 min. When extracted with Triton X-100 prior to formaldehyde fixation, cells were first washed with ice-cold PBS and then treated on ice twice with 0.1% TX-100 mCSK containing multiple protease inhibitors. When stained after DSP-treatment and subsequent chromatin-extraction, cells in the culture chambers were processed as described above prior to formaldehyde fixation. The fixed cells were blocked for 1 hr in 10% normal goat serum in PBS, and incubated overnight at 4°C with primary antibodies diluted in PBS containing 5% normal goat serum. The anti-ORC2, CDC6 and MCM7 rabbit polyclonal antibodies and affinity-purified rabbit antibodies against EB viral BZLF-1 protein as a negative control were used at 1µg/ml. The anti-HA and PCNA murine monoclonal antibodies and control IgG were used at 5µg/ml. The samples were then incubated with secondary antibodies for 1 hr. These were affinity-purified and cross-adsorbed goat anti-rabbit or mouse IgG antibodies conjugated with AlexaFluor 488 or 594 (Molecular Probes). Neither of the control antibodies yielded specific signals in either single-staining or double-staining experiments. All washes after antibody incubation were done with PBS containing 0.1% Tween-20. The samples were mounted in Vectashield (Vector Laboratories). Image acquisition was performed using a Bio-Rad Radiance2000 confocal laser scanning microscope equipped with a PlainApo 60x 1.4 NA oil-immersion objective. Images were processed and assembled in an Apple G3 computer using Adobe Photoshop 5.0.
Establishment of HeLa cells stably expressing 2HA-tagged human ORC1 protein

An oligonucleotide having 5’-HindIII site/Kozak/initiation methionine/2xHA/BamHI site-3’ sequences (5’-AGCTTGCCACCATGTACCCTACGATGTGCCCGATTACGCCG-3’), and its complementary oligonucleotide were synthesized, annealed and introduced into pBluescript to generate pBS2HA. pBSORC1 in which the human ORC1 cDNA is inserted into EcoRV site of pBluescript was provided by Dr. Ohtani. A BamHI site (5’-GGATCC-3’) was introduced into the ORC1 cDNA in place of 5’-ATGGCA-3’ sequence encoding the first methionine and the second alanine by oligonucleotide-directed mutagenesis (QuikChange Site-directed Mutagenesis Kit; Stratagene). The cDNA was then excised with BamHI and cloned into pBS2HA. The sequence of the resultant 2HA-ORC1 cDNA was confirmed by sequencing. The cDNA was excised with HindIII and XbaI and cloned into pTRE2, Tet system expression vector (Clontech). The plasmid pTRE2/2HA-ORC1 was co-transfected with the pTKhyg into HeLa Tet-off cells expressing tTA (Clontech). Two days later, cells were plated in selective medium containing 250μg/ml of hygromycin B and 100 μg/ml of G418.
RESULTS

In vivo cross-linking of HeLa cells with DSP and subsequent biochemical fractionation reveals association of CDC6 with non-chromatin nuclear structure

A significant part of CDC6 protein is associated with nuclear components resistant to nuclease-directed solubilization under relatively physiological conditions (23), whereby about 30% of total chromatin remains (22). Therefore, there are two interpretations; one is that the bound CDC6 is associated with non-chromatin nuclear structures; the other is that they bind to nuclease-resistant specific chromatin region such as heterochromatin. In general, there are three biochemical operations which can define non-chromatin nuclear structures (reviewed in Ref.2). To remove chromatin, all the methods employ digestion with nuclease, but different procedures are used to elute the digested chromatin. One procedure is high-salt extraction (for the nuclear matrix; Refs. 27 and 28). However, some proteins may coagulate under high-salt conditions and be thereby artificially detected as matrix elements, and some proteins that are associated with the matrix in vivo may be stripped by salt extraction. Second is ionic-detergent extraction after stabilizing the matrix by heating or with CuSO4 (for the scaffold; Ref.29). This may also be associated with problems. The mildest procedure is electroelution of the chromatin under relatively physiological conditions (for the nucleoskeleton; Refs. 8 and 9). Our fractionation procedure is a simplified version of this method. With all these methods, the trivial possibility also
remains that insolubility of matrix-related proteins merely results from their non-specific aggregation by exposing cells to nonphysiological buffer. It is also a problem that CDC6 appears to be solubilized with nuclease when cells are processed under hypotonic conditions (30). It is known that “the nuclear matrix” is not seen when nuclei are thus prepared, but it is also very possible that nonphysiological hypotonic conditions alter the native nuclear architecture (2).

We considered that the in vivo protein-protein cross-linking method could overcome these limitations. If the CDC6 proteins are associated with non-chromatin nuclear structures in vivo, they could be covalently cross-linked to the structures by treating viable cells with a permeable protein-protein cross-linker. Consequently, the bound protein would become resistant to chromatin stripping. If they bind to specific chromatin but not to the structures, then they would be extracted with chromatin. We choose DSP as a permeable chemical protein-protein (but not DNA-protein) cross-linker.

In asynchronous HeLa cells, 50-60% of the CDC6 protein is present as a Triton X-100-extractable form and the remainder is bound to nuclei (Fig. 1A; Ref. 23). This ratio was not significantly changed when cells were treated with DSP (Fig. 1A). Therefore, the treatment does not generate non-specific protein aggregation leading to the artificial insolubility. Without cross-linking, almost all of the Triton-insoluble CDC6 was readily extracted with the buffer containing 0.5 M NaCl (Fig. 1A) so that CDC6 is not a classical nuclear matrix protein. In contrast, about 70% of the insoluble CDC6 (40-30%
of total CDC6) became resistant to the salt extraction with DSP treatment (Fig. 1A), indicating that they are covalently cross-linked to salt-insoluble nuclear protein(s). To investigate whether they are linked to chromatin or non-chromatin nuclear structures, the Triton- and salt-extracted nuclei were digested with DNase I followed by further salt extraction. This treatment stripped ~95% of core histones (Fig. 1A) and DNA (not shown). Nevertheless, most of the bound CDC6 remained in the resultant nuclear pellets (Fig. 1A). Immunofluorescence staining after the fractionation showed that the cross-linked CDC6 proteins are indeed present in nuclear dots (Fig. 7). We conclude that significant amounts of CDC6 proteins are associated with non-chromatin nuclear structures in viable HeLa cells. These fractions were also examined for MCM7 proteins, which are present in two different forms, Triton X-100-extractable and Triton-insoluble forms (Fig. 1A; Ref. 22 and 26). The insoluble MCM7 was extracted with 0.5M NaCl as well as CDC6. However, contrary to CDC6 case, only 10-20% of the insoluble MCM7 became resistant to the salt extraction even after cross-linking, and a half of this resistant form was released with chromatin by DNase I digestion (Fig. 1A). The data is consistent with the conclusion obtained with our original fractionation method that most of the MCM complexes are associated with chromatin not affixed to non-chromatin nuclear structures (22).

To further confirm our cross-linking method to be appropriate, we also examined whether MCM3 and MCM7 are cross-linked in vivo. Triton X-100-extractable and the remaining nuclear fractions were
prepared from HeLa cells treated with DSP. The nuclei were then solubilized with SDS and sonication. SDS was also added to the Triton-extractable fractions. These lysates were then immunoprecipitated with anti-MCM7 antibodies. Since MCM hexameric complexes are disrupted with SDS (22), anti-MCM7 antibodies do not co-precipitate MCM3 protein without cross-linking. However, with DSP, MCM3 became efficiently co-precipitated with MCM7 (Fig. 1B). These data further support the utility of our in vivo cross-linking method, and also strengthen the conclusion of MCM3-MCM7 interactions in viable cells and not in the cell extracts.

**CDC6 is associated with non-chromatin nuclear structure even in S phase**

There remains some controversy as to behavior of CDC6 protein during S phase. Exogenously overexpressed CDC6 is translocated into cytoplasm depending on phosphorylation by CDK2 (31, 32). Such relocation is also observed for endogenous CDC6 (23). However, it has been also suggested that nuclear bound CDC6 still remains in S phase (23, 33). To revisit this problem, we examined their localization in HeLa cells by confocal microscope analyses after immunostaining with the anti-CDC6 antibodies. Immunostaining was performed after extracting cells with Triton X-100 prior to formaldehyde fixation as well as after directly fixing cells with formaldehyde.

When formaldehyde-fixed G1 HeLa cells were stained with anti-CDC6 antibodies, nearly homogenous signals were observed in
nuclei (Fig. 2A), as we reported previously (23). In contrast, when cells were Triton-extracted prior to the fixation, focal staining was noted in the nuclei (Fig. 2A). The data together with the data described above (Fig. 1A and 7) suggest that some CDC6 proteins may assemble into foci on non-chromatin nuclear structures. When HeLa cells in early S phase were formaldehyde-fixed and stained with the anti-CDC6, prominent cytoplasmic localization was observed (Fig. 2A), as we reported previously (23). However, focal staining in nuclei was also visible and the nuclear foci were more clearly observed when cells were first Triton-extracted (Fig. 2A).

Data for CDC6 obtained with DSP-treated asynchronous HeLa cells (Fig. 1A) may represent mainly the situation in G1 phase, since approximately 60-70% of asynchronous HeLa cells are in G1 phase. To confirm whether CDC6 is still associated with non-chromatin nuclear structure in S phase, we analyzed hydroxyurea-treated early S phase HeLa cells with the in vivo cross-linking method with DSP. Data shown in Fig. 2B indicate that CDC6 proteins are associated with non-chromatin nuclear structure even in S phase. Again, immunofluorescence staining after the fractionation showed that the cross-linked CDC6 proteins are indeed in the nuclei of S phase HeLa cells (Fig. 8). It is thus conceivable that in HeLa cells, the nuclear CDC6 foci assembled in G1 phase persist even after S phase while the soluble form is translocated, although there may be differences among different cell lines in the efficiency with which CDC6 is exported in S phase. Thus, the CDC6 translocation contributes to prohibition of rereplication, but might be
dispensable. Consistent with this notion, a recent report with Xenopus egg extracts demonstrated that unphosphorylatable mutant CDC6 can support normal DNA replication without its nuclear export (34).

**Human ORC1 proteins are degraded during S phase dependent on proteasome**

It has been reported that total levels of human ORC1 proteins are constant during cell cycle and that they also bind to nuclear components resistant to nuclease-directed solubilization (24, 31). To analyze regulation of ORC1 protein more precisely, we sought to establish HeLa cells stably expressing epitope-tagged ORC1 protein. We constructed a vector encoding tandem HA epitope-tagged human ORC1 protein, and established HeLa cells stably expressing 2HA-ORC1. The following studies were done with HO-2, a representative HeLa line expressing 2HA-ORC1.

We first investigated the levels of the 2HA-ORC1 proteins during the cell cycle. A clear decrease was observed in the S phase cells (Fig. 3). This is strikingly different from the previously reported finding that ORC1 protein levels are relatively constant during the cell cycle (24, 31). The reason for the difference is not clear at present. Since we use a HA-tagged system, the specificity of results with immunoassays could be higher. When MG132, a peptide aldehyde that binds and inactivates 26S proteasomes (35), was added to the culture, the S phase degradation of ORC1 was decreased and a ladder of ORC1 bands with much retarded mobility, presumably due to
ubiquitinated intermediates, was detected (Fig. 3). No obvious difference in MCM7 or ORC2 protein levels was observed among the samples (Fig. 3). During preparation of this paper, it was reported on the basis of work with monospecific anti-ORC1 antibodies that ORC1 is dissociated from chromatin during S phase and might be degraded in HeLa cells (36). Together, the available data suggest that human ORC1 proteins are degraded during S phase dependent on the ubiquitin-proteasome system.

In vivo cross-linking with DSP reveals association of ORC1 with non-chromatin nuclear structure

Previously it was reported that ORC1 protein also binds to nuclease-resistant structures throughout the cell cycle (24). We revisited this point with the HO-2 HeLa cells and our original fractionation method without DSP (22, 23). Most 2HA-ORC1 proteins were found to bind to nuclease-resistant structures in asynchronous HO-2 cells (Fig. 4). In vivo DSP cross-linking of asynchronous HO-2 cells and subsequent biochemical fractionation further showed 2HA-ORC1 association with non-chromatin nuclear structure (Fig. 5). Immunofluorescence staining after the fractionation with anti-HA antibody further showed foci in the nuclei (Fig. 7). Taken together, these data indicate that ORC1 is associated with non-chromatin nuclear structure during G1 phase.
Some ORC2 proteins are associated with nuclease-resistant nuclear structures, like ORC1 but the remainder bind to nuclease-sensitive chromatin

Previous studies have suggested differences in biochemical and expression profiles between ORC1 and the other ORC subunits in mammalian cells (for details see the Discussion). Therefore, we investigated biochemical localization of ORC2 proteins with our original fractionation method without DSP (22, 23). As shown in Fig.4, about half of the ORC2 proteins were resistant to Triton X-100 extraction, and half of the Triton-insoluble ORC2 was release with DNase I digestion. The data suggest that at least some ORC2 proteins bind to nuclease-sensitive chromatin without any ORC1 association. However, it is also notable that a significant part of ORC2 proteins remain in the form associated with nuclease-resistant structures. This fraction was extracted with 0.5M NaCl (data not shown). The distribution pattern of ORC2 proteins remained unchanged after cells entered into S phase (data not shown). We also analyzed ORC2 localization with DSP cross-linking. Unexpectedly, much of the Triton-insoluble ORC2 fraction then became resistant to DNase I-mediated solubilization (data not shown). Therefore, it could be that chromatin-associated ORC2 fraction also interacts to non-chromatin structure with a low affinity that is disrupted by 100mM NaCl contained in mCSK buffer but is cross-linked with DSP. Alternatively, the conditions of DSP cross-linking may be inappropriate for analyses of ORC2 proteins.
ORC1 physically interacts with ORC2 in vivo

Using HO-2 cells, we assessed physical interactions of 2HA-ORC1 with ORC2, CDC6 or MCM7. To extract these efficiently from nuclei, 0.5M NaCl is required in the extraction buffer. However, physical interactions among them are disrupted under this condition. Therefore, nuclear extracts were prepared with buffer containing 0.25M NaCl, although results in partial solubilization. The extracts were immunoprecipitated with the rat anti-HA antibody. ORC2 was efficiently co-precipitated with 2HA-ORC1 (Fig. 6). We conclude that ORC1 relatively tightly interacts with ORC2 possibly on non-chromatin nuclear structures. Neither CDC6 nor MCM7 co-precipitated with 2HA-ORC1. Even if chemical cross-linking was performed for viable cells or Triton-extracted nuclei before immunoprecipitation, we could not observe co-precipitation of CDC6 or MCM7 with 2HA-ORC1 (data not shown). In addition, we could not observe co-precipitation between ORC2 and CDC6 (data not shown).

Relative organization of ORC1, ORC2, CDC6 and MCM proteins in G1 phase nuclei assessed by immunostaining and confocal microscopic analysis after DSP cross-linking and chromatin-extraction

To gain further insight into the nuclear organization of ORC1, ORC2, CDC6 and MCM7, we examined their localization in DSP-cross-linked and chromatin-extracted G1 phase HeLa cells by confocal microscope analyses after double-immunostaining. The anti-HA is a mouse monoclonal and the other antibodies are rabbit
We stained HO-2 HeLa cells simultaneously for 2HA-ORC1 and ORC2, CDC6 or MCM7. 2HA-ORC1 proteins were found in nuclear dots. The data, in combination with the findings of biochemical fractionation (Figs. 4 and 5), suggest that multiple ORC1 proteins assemble into foci based on non-chromatin structures. Most of 2HA-ORC1 foci were found to colocalize with ORC2, while there was also ORC2 staining not associated with 2HA-ORC1 (Fig. 7). The staining patterns further support the notion suggested by the biochemical data (Figs. 4 and 6) that ORC1 interacts with ORC2 on non-chromatin structures while some ORC2 proteins exist without any ORC1 association.

Immunostaining with the anti-CDC6 antibodies revealed focal nuclear staining in DSP-cross-linked and chromatin-extracted G1 phase HeLa cells (Fig. 7). In combination with the results of biochemical fractionation (Fig. 1), this suggests that multiple CDC6 proteins also assemble into foci based on non-chromatin structures. However, only a partial colocalization was observed between 2HA-ORC1 and CDC6 foci (Fig. 7).

It has been reported that anti-MCM antibodies give homogenous nuclear staining in G1 phase, even after extracting cells with non-ionic detergents (data not shown; ref. 17, 37). This, together with the findings that the number of MCM molecules bound to chromatin is several to ten times that of bound ORC1 or CDC6 (17, 23, 24, 31), indicates that MCM hexamers are widely distributed on chromatin rather than in focal regions like ORC1 and CDC6. On the other hand, after DSP cross-linking and subsequent extraction, about 10% of
chromatin-bound MCM proteins remain in the nuclei (Fig. 1A). To test whether the residual MCM proteins are close to the ORC1 foci, we performed double-immunostaining experiments. Most of 2HA-ORC1 foci were found to colocalize with MCM7 in DSP-treated and chromatin-extracted G1 phase HeLa cells (Fig. 7). Therefore, at least some proportion of chromatin-bound MCM complexes may be loaded near ORC1 foci. MCM7 staining not associated with 2HA-ORC1 staining was also apparent, which may represent residual chromatin-bound MCM complexes far from ORC1 foci.

**Relationship between PCNA foci and ORC or CDC6 foci in early S phase cells**

PCNA is one of the major components of replication foci constructed based on non-chromatin nuclear structures (8, 9). In aphidicolin-arrested early S phase HeLa cells, PCNA foci may be built up near initiation sites of early replicons. We examined relationship between PCNA foci and ORC or CDC6 foci under these conditions by double-immunostaining and confocal microscope analyses after DSP-treatment and chromatin-extraction. Only a partial colocalization with many non-overlapping foci was observed between PCNA and ORC2 or PCNA and CDC6 foci (Fig. 8). ORC2 and CDC6 foci not associated with PCNA foci might correspond to replicons yet unfired. However, the existence of PCNA foci not associated with ORC2 or CDC6 foci suggests that initiation of DNA replication might not necessarily occur near ORC or CDC6 foci.

It is known that MCM proteins do not colocalize at site of newly
synthesized DNA at least from immunostaining evidence (37). Also in our hand, PCNA foci did not colocalize with MCM7 in aphidicolin-arrested early S phase HeLa cells when examined by double-immunostaining after DSP-treatment and chromatin-extraction (data not shown).
DISCUSSION

Organization and cell cycle regulation of ORC in mammalian cells

Six proteins, ORC1 to 6, form a stable heterohexameric complex in budding yeast (reviewed in Ref.16). Such a holocomplex has also been detected in DNA replication systems with Xenopus egg extracts or Drosophila embryo extracts (21, 38), both of which represent the embryonic cell cycle.

In mammalian somatic cells, although the homologues for the six subunits have been identified, their mode of complex formation seems still somewhat controversial. It was first reported that T7-tagged ORC1 co-immunoprecipitate with anti-ORC2 antibodies when transiently overexpressed (39). On the other hand, a series of studies by Dutta et al. suggest that ORC2 to 5 make stable complexes while ORC1 is difficult to detect in the complexes (40-43). It is also notable that expression of ORC2 to 5 is cell growth-independent, while ORC1 expression is driven by E2F (31, 40-44). Taking these findings and our present data together, one can draw the following picture. On non-chromatin nuclear structures, ORC1 forms a complex with ORC2 (Fig. 4, 6 and 7) and probably ORC3, 4 and 5, probably functioning in DNA replication. ORC6 might also be included in the complex (43). In addition, there is another subcomplex containing ORC2 but lacking ORC1, which binds to nuclease-sensitive chromatin (Fig.4) and may exist even in non-proliferating cells. It is not clear whether such subcomplexes also play a role in DNA replication, but more probably they may act in other function(s) (45).

Our present data suggest that ORC1 protein is degraded via
ubiquitin-proteasome system after cells entering into S phase (Fig.3). In fission yeast, phosphorylation of Cdc18, a homologue of CDC6, by the CDK leads to its rapid degradation, which contributes to suppression of rereplication (reviewed in Ref.16 and 17). However, in mammalian cells, CDC6 protein levels are unchanged after S phase (Fig.2; Ref.23). Rather ORC1 degradation after S phase may contribute to prohibition of rereplication in mammalian cells.

**Association of CDC6 with ORC in mammalian cells**

CDC6 as well as ORC1 and ORC4 are related to RFC at the amino acid sequence level (46). Therefore, ORC and CDC6 may cooperatively load MCM complexes. In budding yeast, CDC6 binds to origins in vivo and in vitro probably via its physical interaction with ORC (47, 48). In the Xenopus egg extract system, although CDC6 binds to chromatin depending on ORC binding, how the latter recruits CDC6 is unclear; so far significant physical interaction has not been observed, either in the extracts or when those assembled on chromatin were analyzed (20, 21). Therefore, their physical interaction, if any, may be unstable. In mammalian cells, it is reported that CDC6 is co-purified with ORC1 through several column chromatographic steps, and that they can physically interact in vitro (31). However, so far we could not detect any co-immunoprecipitation between CDC6 and ORC1 or ORC2 (Fig.6), so that the situation is similar to that in the egg extract system. Furthermore, although both ORC1 and CDC6 interact with non-chromatin nuclear structures (Figs.1 and 5), our double-immunostaining experiments showed only a partial
colocalization (Fig. 7). The loading of MCM proteins onto chromatin occurs during late mitosis in cycling cells, when ORC proteins are abundant but the levels of CDC6 are low (30). Therefore, only transient and weak interaction between ORC and CDC6 proteins on non-chromatin structures might be sufficient for their function to load MCM.

MCM complexes are mainly loaded onto chromatin not affixed to non-chromatin nuclear structures

In budding yeast, chromatin immunoprecipitation assays suggest that MCM proteins associate with chromatin around origins (47). MCM is required not only for initiation but also for the elongation step (49). One notion for MCM function is that it acts as a kind of replicative helicase, moving from an origin with elongation machinery (50, 51). Also in Xenopus egg extract system, it is assumed that MCM complexes are loaded near ORC/CDC6 binding site. In this case, multiple MCM complexes may be loaded on each ORC site (52), which appears also to be the case in mammalian somatic cells (23). Whatever, MCM may not bind to chromatin via its physical interaction with ORC/CDC6 (23, 52, 53).

We previously suggested that most MCM complexes bind to chromatin not affixed to non-chromatin nuclear structures (23). Our present data further indicate that while a small proportion of MCM complexes are loaded onto chromatin regions near ORC foci, most of them are more widely distributed (Fig. 7). Given that multiple MCM complexes are loaded at single ORC site, how do they function in DNA
replication? Chromatin loading of MCM complexes is strictly controlled, reloading being prohibited during S phase by CDK activity (reviewed in Refs.16 and 17). Therefore, if the MCM complex is loaded only near an origin and should move as a replicative helicase over long distances, replication fork may be arrested once it is accidentally dissociated. Multiple MCM complexes might act as a failsafe mechanism to overcome such a problem. It is unclear why the distribution pattern of MCM is apparently different between budding yeast and mammalian cells.

**Possible relationship between the organization of prereplication chromatin and origin specification in higher eukaryotic cells**

In higher eukaryotes, it has been suggested that the DNA is periodically attached to the nuclear matrix at specific sequences, matrix-attachment regions (reviewed in Ref.10). A recent study revealed that in the early embryonic cell cycle, the DNA is randomly attached to the matrix, but during the midblastula transition, the time when zygotic transcription is activated, sequences associated transcription become specifically attached (54). The timing is also when the sequence requirement for replication origin activity transits from apparently random to more specific (55). Therefore, given that chromatin regions affixed to non-chromatin nuclear structures could more easily access ORC, this could be one explanation for such a developmental change in origin specification.

Although replication origins are specified in somatic cells, the situation is not simple. Studies of replication origin activity...
in the DHFR domain indicate that replication can initiate at any of multiple potential sites scattered throughout the 55kb intergenic region in this domain, with two subregions being preferred, termed ori-β and ori-γ (reviewed in Refs. 56 and 57). In addition, despite the preference, ori-β is not essential for the origin activity of the region (58). Again, our proposed model could provide an explanation for the complexity. In the egg extract system, once MCMs are loaded, ORC/CDC6 is no longer required for DNA replication (52, 53). Therefore, given that each of the sites onto which the multiple MCM complexes are loaded can act as one potential initiation site, this architecture could represent the initiation zone. If initiation of replication occurs only at MCM complexes near ORCs, then PCNA foci would be detected only near ORC foci in the early S phase. However, this does not appear to be the case (Fig.8), suggesting that once MCM complexes are loaded onto chromatin, initiation might also occur apart from the ORC sites. Preferred initiation sites might be those that are free from nucleosomes and thus locations onto which MCM are preferentially loaded. Alternatively, they might be sites which can easily be unwound. For the further understanding, it is very important to precisely determine distribution patterns of initiation proteins on mammalian cell replicons, for example by chromatin immunoprecipitation assays.
ACKNOWLEDGMENTS

We thank Dr. K. Ohtani (Tokyo Medical and Dental University) for providing pBSORCl, and T. Yoshida, C. Yamada, Y. Nishikawa and K. Matsuguchi for technical assistance.
REFERENCES


The abbreviations used are: ORC, origin recognition complex; DSP, dithiobis-succinimidylpropionate; PBS, phosphate-buffered saline.
FIGURE LEGENDS

Fig. 1.
Biochemical analyses of localization of CDC6 and MCM7 proteins in HeLa cells treated with DSP in vivo. (A) Triton X-100-extractable supernatants (TX-100 S) and extracted nuclei (TX-100 P) were prepared from asynchronous HeLa cells treated with or without DSP. The nuclei were extracted with buffer containing 0.5 M NaCl, and the salt-extracts (NaCl S) and the nuclear pellets (NaCl P) were separated. The Triton- and salt-extracted nuclei were then treated with DNase I followed by salt extraction to obtain solubilized chromatin fractions (DNase S) and insoluble nonchromatin nuclear pellets (DNase P). These fractions were immunoblotted with anti-CDC6 or MCM7 antibodies. The samples were also subjected to SDS-PAGE followed by Coomassie Blue staining for detection of core histones. (B) Detection of protein-protein interactions among MCM proteins by in vivo DSP cross-linking. Triton X-100-extractable fractions and extracted nuclear pellets were prepared from asynchronous HeLa cells treated with or without DSP. SDS was added to the extractable fractions. The nuclei were solubilized with SDS and sonication. These lysates were immunoprecipitated with anti-MCM7 antibodies, and the immunoprecipitates (IP) and the lysates (input) were immunoblotted with anti-MCM7 or MCM3 antibodies.

Fig. 2.
**Subcellular localization of CDC6 proteins during S phase.** (A) Confocal microscopic analyses of localization of CDC6 proteins in HeLa cells in G1 or early S phase. Cells in mid to late G1 phase or hydroxyurea-treated early S phase were fixed with formaldehyde directly or after extraction with Triton X-100, and then immunostained with anti-CDC6 or control antibodies (Alexa; green). The samples were further treated with propidium iodide for DNA staining (PI; red). (B) Biochemical analyses of localization of CDC6 proteins in early S phase HeLa cells using in vivo cross-linking with DSP. Hydroxyurea-treated early S phase cells were analyzed as in Fig.1A.

**Fig. 3.**

**Human ORC1 proteins are degraded during S phase dependent on proteasomes.** Asynchronous (AS), hydroxyurea-treated early S phase (HU), or nocodazole-treated G2/M phase (NOC) HO-2 HeLa cells expressing 2HA-ORC1 were incubated with or without 20µM MG132, a peptide aldehyde that binds and inactivates 26S proteosomes, for further 8hr. Whole cell lysates were then prepared from them and analyzed by immunoblotting with anti-HA, anti-MCM7 or anti-ORC2 antibodies.

**Fig. 4.**

**Biochemical analyses of localization of ORC1 or ORC2 proteins in HeLa cells.** Triton X-100-extractable supernatants (Triton S) and extracted nuclear pellets (Triton P) were prepared from asynchronous HO-2 HeLa cells. The extracted nuclei were further treated with
DNase I to obtain DNase I-solubilized supernatants (DNase S) and remaining DNase-resistant nuclear pellets (DNase P). These fractions were immunoblotted with anti-HA and anti-ORC2 antibodies. As a control protein released by DNase I digestion, MCM7 was also detected in the fractions. Note that in vivo cross-linking with DSP was not used in these experiments.

**Fig. 5.**

**Biochemical analyses of localization of ORC1 proteins in HeLa cells using in vivo cross-linking with DSP.** Asynchronous HO-2 HeLa cells were treated with or without DSP and processed as in Fig.1A. The fractions were immunoblotted with anti-HA antibodies. The samples were also subjected to SDS-PAGE followed by Coomassie Blue staining for detection of core histones.

**Fig. 6.**

**Co-immunoprecipitation of ORC2 with ORC1.** Nuclear extracts were prepared from HO-2 cells and control parental cells with buffer containing 250mM NaCl, and immunoprecipitated with the anti-HA rat monoclonal or control antibody. The immunoprecipitates (IP) and the extracts (input) were immunoblotted with the anti-HA mouse monoclonal antibody and the anti-ORC2, CDC6 and MCM7 rabbit polyclonal antibodies.

**Fig. 7.**
Relationship between ORC1 and ORC2, CDC6 or MCM proteins in G1 phase HeLa cell nuclei assessed by immunostaining and confocal microscopic analyses after DSP cross-linking and chromatin-extraction. G1 phase HO-2 cells were treated with DSP and then chromatin-extracted as described in the “Experimental Procedures”. The processed cells were fixed with formaldehyde and then immunostained simultaneously for 2HA-ORC1 (red) and ORC2, CDC6 or MCM7 (green).

Fig. 8.

Relationship between PCNA and ORC2 or CDC6 proteins in aphidicolin-arrested early S phase HeLa cell nuclei assessed by immunostaining and confocal microscopic analyses after DSP cross-linking and chromatin-extraction. Aphidicolin-arrested HO-2 cells were treated with DSP and then chromatin-extracted as for in Fig.7. The processed cells were fixed with formaldehyde and then immunostained simultaneously for PCNA (red) and either ORC2 or CDC6 (green).
Fig. 1
**Fig. 2**

A. 

<table>
<thead>
<tr>
<th>Phase</th>
<th>Formaldehyde</th>
<th>Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDC6</td>
<td>Alexa</td>
<td>PI</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. 

- TX-100
- 0.5M NaCl
- DNase(-)
- DNase(+)
- No cross-link

- CDC6
- Histones

DSP
Fig. 3
Fig. 4
Fig. 6
Nuclear organization of DNA replication initiation proteins in mammalian cells
Masatoshi Fujita, Yukio Ishimi, Hiromu Nakamura, Tohru Kiyono and Tatsuya Tsurumi

J. Biol. Chem. published online January 4, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111398200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2002/01/04/jbc.M111398200.citation.full.html#ref-list-1