Human Minichromosome Maintenance Proteins and Human Origin Recognition Complex 2 Protein on Chromatin

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Minichromosome maintenance (Mcm) proteins and the constituents of the origin recognition complex (Orc) are essential components of the eukaryotic replication initiation apparatus. Published evidence strongly suggests that the binding of Mcm proteins to chromatin is contingent upon the prior binding of Orc proteins. Here we use two different approaches to investigate the presence of the human ORC2 protein and of Mcm proteins on chromatin of Hela cells in various cell cycle phases. First, we mobilized chromatin-bound proteins by micrococcal nuclease and analyzed the resulting digestion products by sucrose gradient centrifugations. Under digestion conditions when Mcm proteins were almost entirely released from chromatin, ORC2 protein was found to be associated with chromatin fragments containing several hundred base pairs of DNA. Second, we used an in vivo cross-linking procedure to covalently link Mcm proteins and ORC2 to DNA by short exposure of intact HeLa cells to formaldehyde. Specific immunoprecipitations revealed that cross-linked nucleoprotein fragments carried either Mcm proteins or ORC2 protein, but not both. Based on the lengths of the DNA fragments in immunoprecipitates, we estimate that the distance between chromatin-bound ORC2 protein and chromatin-bound Mcm proteins must be at least 500–1000 base pairs in HeLa cells.

Considerable progress has recently been made in describing the components of the machinery required for the initiation of eukaryotic genome replication. Much of the original work was performed studying yeast mutants that had lost the ability to perform defined steps in the progression of proliferating cells through the late G1 phase or early S phase of the cell cycle. Genetic studies were complemented by cell biological and biochemical experiments leading to the identification of yeast DNA elements, known as ARS (autonomously replicating sequences), which serve as origins of replication, and of the six-Mcm elements, bound to replication start sites, recruit the Cdc6-encoded protein (Cdc6p) as a requirement for the subsequent binding of Mcm proteins (8–14). Other proteins may also be involved in the formation of replication-competent chromatin (15–17). Bound Cdc6p is inactivated and degraded at the beginning of S phase, whereas Mcm proteins are released from their chromatin sites during the course of DNA replication (reviewed in Refs. 2–4). Consequently, newly replicated sections of chromatin are free of Mcm proteins and can therefore not re-replicate during the same cell cycle (18–20).

Mcm proteins occur in high copy numbers in nuclei of human cells. They appear to be either free in the nucleoplasm or bound to chromatin. Although not yet formally proven, it can be assumed that the binding of mammalian Mcm proteins to chromatin is also contingent upon the previous binding of Orc proteins and Cdc6 protein. The fraction of structure-bound mammalian Mcm proteins is highest at the beginning of S phase, but gradually decreases during progression of replication (21–23). Released nucleoplasmic Mcm proteins are found as large multicomponent complexes, composed of the six known members of the Mcm protein family. This complex has the tendency to disintegrate into more stable subcomplexes of which one is a dimer of the MCM3 protein (MCM3p) and the MCM5 protein (MCM5p), while the second is a trimer of proteins MCM4 (MCM4p), MCM6 (MCM6p), and MCM7 (MCM7p). The sixth member of the family, human protein MCM2 (MCM2p), is usually found to be loosely associated with these subcomplexes (24–26).

The present study was performed to investigate the mode of binding of Mcm proteins and ORC2p, a member of the origin recognition complex, on human chromatin. We prepared chromatin from proliferating human HeLa cells using low ionic strength conditions in the presence of the nonionic detergent Nonidet P-40. As first shown by Hancock (27), the nuclear envelope is removed by this procedure, while the general form and the ultrastructural characteristics of chromatin appear to remain largely unchanged. The maintenance of form in this structure allows biochemical manipulations such as pipetting and low speed centrifugations for a separation of structure-bound from soluble nuclear material. We have used these prep-
arations before to investigate the chromatin-associated fraction of MCM6p, and found that the protein can be released from chromatin by a treatment with micrococcal nuclease (26). We expected to find ORC2p and Mcm proteins on the same chromatin fragments, but our results showed that this is not the case and imply that Mcm proteins and ORC2p are located on different chromatin sites. This conclusion was supported by results obtained through formaldehyde cross-linking in vivo.

EXPERIMENTAL PROCEDURES

Chromatin from Synchronized HeLa Cells—As previously demonstrated (25, 34) and confirmed in the present study (not shown), about one half of nuclear Mcm proteins is free in the nucleosol of prereplicative HeLa cells, while the second half is bound to chromatin. To investigate chromatin-bound proteins we used the classic method of Hancock (27) for the preparation of chromatin from HeLa cells. The method involves a treatment of washed cells with buffers of very low ionic strengths in the presence of the nonionic detergent Nonidet P-40. The antibodies react with the six human Mcm proteins as described previously (25, 31, 32). Affinity-purified antibodies were used for immunostaining with the indicated Mcm-specific antibodies. C, as in B, except that the blots were immunostained with ORC2-specific antibodies.

RESULTS

Chromatin Structure and Cross-linking—Chromatin was prepared using buffers with very low ionic strengths in a modification of the procedure originally described by Hancock (27). Cells on plates were washed three times with 30 ml each of 0.25 mM EDTA and centrifuged at 5 min at 700 × g) and washed again with 20 ml of 0.25 mM EDTA. The pellet was then resuspended in 4 ml of 0.25 mM EDTA plus 2 mM P-40 and centrifuged through a 20-ml sucrose cushion (0.1 M sucrose in 0.5 mM Tris-HCl, pH 8.5) for 10 min at 4000 × g. Pellets were suspended in 2 ml 0.25 mM EDTA and again centrifuged through a sucrose cushion as above to remove the remaining traces of Nonidet P-40. The final pellet was resuspended in 0.25 mM EDTA to give a final concentration corresponding to 500 μg of DNA/ml.

Immunofluorescence studies with nuclei from cultured mammalian cells had previously shown that Mcm proteins dissociate from their chromatin binding sites when cells progress through the S phase of the cell cycle (21–23). Thus, if our chromatin preparations reflect the situation in vivo, we would expect to see relatively high amounts of Mcm proteins on isolated chromatin from early S phase cells, and decreasing amounts on chromatin from later stages of the S phase. To address this point, we prepared chromatin from HeLa cells, arrested by a double-thymidine block at the transition between G1 and S phase, as well as from cells at various times after release of the arrest. Synthesis of DNA, as determined by the incorporation of labeled DNA precursors, started between 2 and 3 h after removal of thymidine and then continued for approximately 6 h. The end of the S phase partially overlapped with the beginning of mitoses, probably reflecting variations in individual cell cycle times (Fig. 1A). Preparation of chromatin during mitosis did, of course, not produce the spherical chromatin structures as described above for interphase chromatin, but yielded an unstructured dense chromatin mass, which could nevertheless be precipitated by low speed centrifugation for further investigations.

Using specific antibodies in immunoblot experiments, we determined that Mmc proteins on chromatin decreased during S phase, but rapidly bound to chromatin again after completion of mitosis (Fig. 1B). However, ORC2p remained bound to chromatin during all stages of the HeLa cell cycle including mitosis (Fig. 1C). Similar observations have been reported before for Orc proteins of yeast (12, 13, 17, 36) and of Xenopus, although...
bound Xenopus Orc proteins can be detected only on anaphase and telophase chromosomes, but not on metaphase chromosomes (8). The synchronization of the HeLa cell cycle is not precise enough to decide whether ORC2p remained on human metaphase chromosomes or not.

Aphidicolin, an inhibitor of replicative DNA polymerases, has been reported to prevent the loss of Mcm proteins from Xenopus chromatin (18, 37). In agreement with these earlier findings, we could also show for HeLa cells that, when aphidicolin was added to synchronized cells between 1 and 3 h after start of growth, Mcm proteins did not dissociate and remained bound to chromatin during the following 7 h (not shown).

In summary, the experiment with isolated chromatin mirrors the cycle of Mcm dissociation and reassociation, as described previously for HeLa cells in vivo (23), and shows furthermore that chromatin-bound ORC2p does not dissociate in proliferating human cells. The experiment is also of interest for technical reasons because it excludes the possibility that Mcm proteins may be artifactually trapped in chromatin pellets.

**Treatment with Micrococcal Nuclease**—We digested isolated chromatin with micrococcal nuclease, an enzyme that preferentially cleaves the linker DNA between adjacent nucleosomes (reviewed in Ref. 40). Mcm proteins were released together with fragmented DNA in a reaction which depended on the amounts of enzyme used (Fig. 2A, panel a) or on the time of incubation (Fig. 2B, panel a) (26). Proteins remained stably bound to chromatin incubated in enzyme buffer without micrococcal nuclease.

ORC2p was also mobilized from chromatin by nuclease treatment (panels b in Fig. 2, A and B) at a rate that paralleled the release of chromatin fragments (panel c in Fig. 2B). In control experiments, we demonstrated that a treatment of chromatin with RNase A did not mobilize Mcm proteins or ORC2p (not shown).

Micrococcal nuclease degrades chromatin to fragments whose sizes depend on the extent of digestion. We investigated whether Mcm proteins and ORC2p remained bound to chromatin fragments. For this purpose, chromatin was prepared from HeLa cells, arrested by a double thymidine block at the G1/S phase transition of the cell cycle, as well as from cells at 5 and 13 h after the removal of excess thymidine (see Fig. 1). The chromatin preparations were treated in parallel with micrococcal nuclease, and the chromatin digestion products were sedimented through sucrose gradients in buffers with low salt concentration. In Fig. 3 we show the gradient analysis of chromatin digests from HeLa cells arrested at the boundary between G1 and S phase. But very similar data were obtained analyzing sucrose gradients with chromatin digests from S phase cells (5 h after release from the double thymidine block) and from G2 phase cells (13 h after release) (not shown).

The positions of chromatin fragments were determined by their absorbance of ultraviolet light at 254 nm and confirmed by a gel electrophoretic analysis of histones. We could thus determine the positions in the gradient of chromatin fragments bearing one, two, or more nucleosomes (Fig. 3A). Immuno blotting experiments showed that MCM3p appeared at two positions, as a free monomeric protein, sedimenting at about 4 S, and as a larger complex together with MCM5p, sedimenting at about 12 S, just in front of the mononucleosomal peak (Fig. 3B). MCM5p and MCM7p in the 12 S peak could be immunoprecipitated with MCM3-specific antibodies (not shown) and must therefore be closely associated (24).

We have used specific antibodies directed against MCM6p, MCM4p, and MCM7p in immunoblot experiments to determine the distribution of these proteins in the gradient. A minor portion of MCM6p appeared as free protein sedimenting at 4 S, much like free MCM3p, but the remaining and larger part of MCM6p was distributed together with MCM4p over many of the faster sedimenting fractions in the gradient with a maximum just behind the peak of mononucleosomes (Fig. 3B). Holthoff et al. (26) have described before this distribution of chromatin-derived MCM6p and noted that MCM6p, but also MCM4p and MCM7p, tend to aggregate when they are released from chromatin under the low salt conditions used. The distribution in the gradient of MCM7p partially overlaps the MCM3p/MCM5p peak as well as the MCM4p/MCM6p peaks.

We also determined the distribution of ORC2p in the sucrose gradient, and found that its sedimentation properties were clearly different from those of the Mcm proteins (Fig. 3C). In fact, its high sedimentation rates suggested that ORC2p remained associated with fragmented chromatin in contrast to the Mcm proteins, which sedimented largely independent of nucleosomes. To further investigate this possibility, we performed sucrose gradient centrifugations of chromatin frag-
ments obtained after short (5 min) and after longer (40 min) treatment with micrococcal nuclease. As expected, the fraction of oligonucleosomes decreased while the fraction of mononucleosomes increased with digestion times (panels a in Fig. 4). After a short digestion time, ORC2p appeared as a broad peak together with oligonucleosomes (panel b in Fig. 4A), but, after longer digestion, ORC2p was found in a slower sedimenting complex between the mononucleosomal and the dinucleosomal peak (panel b in Fig. 4B). This result indicates that continued nuclease treatment removed any excess DNA that remained bound to ORC2p after short digestion.

More extended nuclease digestion using higher enzyme concentrations or longer incubation times did not further change the sedimentation rate of the ORC2p-containing complex (not shown). The ORC2p-containing complex most likely included the other Orc proteins as well because Gavin et al. (35) could immunoprecipitate ORC2p in association with other human Orc proteins. The six-membered Orc has a combined molecular mass of approximately 400 kDa (35) and could have a sedimentation coefficient of 13 S, like the ORC2p-containing peak in Fig. 4B (estimated relative to the sedimentation coefficient of mononucleosomes; 10–11 S).

For comparisons, we also determined the distribution of MCM3p in the sucrose gradient of Fig. 4. As already shown above, one part of MCM3p was recovered as a monomeric protein at 4 S, while another part sedimented at about 12 S, both after short and long digestion times (panels b of Fig. 4). The 12 S peak of MCM3p exhibited a leading shoulder after short, but a more symmetric distribution after longer, digestion times, probably suggesting that residual pieces of DNA, which still remained on MCM3p after brief nuclease treatment, were later degraded. Using the appropriate antibodies, we found again
(see Fig. 3) that MCM5p was present in the 12 S peak, but not in the 4 S peak of MCM3p (not shown, but see Holthoff et al. (26) for a detailed description of Mcm proteins on micrococcal nuclease digests of chromatin).

To summarize the data of this section we note that rather short digestion times were sufficient to mobilize chromatin-bound Mcm proteins (Fig. 2), suggesting that Mcm proteins may reside on chromatin sites which are more open to nuclease attack than bulk chromatin. Furthermore, sucrose gradient analyses of digestion products revealed that most Mcm proteins sedimented largely independent of nucleosomes whereas ORC2p remained bound to oligosomal chromatin fragments. This indicates that ORC2p and Mcm proteins are probably not located at closely adjacent sites. To support this conclusion we resorted to an entirely different experimental approach, namely in vivo cross-linking of chromatin proteins to DNA.

**Cross-linking of Chromatin Proteins**—We have used a procedure, described in detail by Göhring and Fackelmayer (29), which involves a treatment of growing HeLa cells with 1% formaldehyde. Nuclei from formaldehyde-treated cells were lysed in sodium Sarkosyl and centrifuged through a CsCl step gradient to remove the proteins that were not cross-linked to DNA. The nucleoprotein complex was then sheared to give fragments with DNA of 500–1000 base pair lengths, which were recentrifuged in a CsCl equilibrium gradient until the remaining nucleoprotein fragments had accumulated at the buoyant density characteristic for their protein/DNA ratios (29). Covalent linkages between proteins and between proteins and DNA were cleaved by boiling in electrophoresis sample buffer containing β-mercaptoethanol and sodium dodecylsulfate (39). Following this procedure, Burkhart et al. (24) had previously shown that MCM3p was present in the cross-linked nucleoprotein complexes recovered after CsCl equilibrium centrifugation.

To demonstrate the efficiency of the cross-linking procedure, we first performed an experiment exposing growing HeLa cells to formaldehyde for increasing lengths of time. The recovered nucleoprotein complexes were investigated by denaturing polyacrylamide gel electrophoresis and silver staining showing that significant amounts of histones were covalently linked to DNA already after 2–4 min cross-linking time while non-histone proteins appeared at later times in the nucleoprotein complexes (Fig. 5A). Mcm proteins were among the non-histone proteins as shown by immunoblotting with antibodies that recognize the most conserved region of all Mcm proteins including the central DEFD motif (Fig. 5B). Since MCM3p and MCM6p are not well separated by standard polyacrylamide gel electrophoresis (26), the blots used in Fig. 5B were stripped and reprobed with MCM6-specific antibodies (Fig. 5B).

Cross-linking of Mcm proteins to DNA was detectable after 8 min of treatment with formaldehyde, but continued for up to 30 min, whereas cross-linking of ORC2p began as early as 4 min and was essentially complete after 8 min cross-linking time (Fig. 5C). As a control, we analyzed for the scaffold attachment factor A (SAF-A) (41) in cross-linked nucleoprotein and found that this protein was efficiently coupled to DNA after only 2–4 min (Fig. 5D). The different rates at which proteins were cross-linked to DNA may reflect their position in chromatin. Thus, SAF-A is known to be in immediate contact with DNA and cross-linked to DNA almost as efficiently as histones, but ORC2p may be bound to DNA via other members of the origin recognition complex (42) and therefore cross-linked later than SAF-A, but earlier than Mcm proteins, which are probably tethered to DNA via other proteins, possibly including histones (43).

We treated HeLa cells at different cell cycle stages (Fig. 6A) for 8 min with formaldehyde and prepared cross-linked nucleoprotein complexes as just described. Cross-linked nucleoprotein complexes were immunoprecipitated using several specific antibodies. The results show that immunoprecipitated nucleoprotein fragments, obtained through Mcm-specific antibodies, carried Mcm proteins, but not ORC2p; and precipitates, obtained through ORC2-specific antibodies, carried ORC2p, but not Mcm proteins (Fig. 6A). The diffuse bands in the ORC2 lane in panel a of Fig. 6B have not been characterized, but their relation to one of the Mcm proteins could be excluded because the bands did not react with individual Mcm-specific antibodies (not shown). Since 8 min of cross-linking time was sufficient to covalently link essentially all detectable ORC2p to DNA (Fig.

**Fig. 5. Cross-linking of nucleoprotein.** Nuclei from HeLa cells, treated for the indicated times with 1% formaldehyde, were lysed. The resulting nucleoprotein complexes were purified by two consecutive CsCl centrifugations. A, denaturing polyacrylamide gel electrophoresis and silver staining. B, immunoblotting with DEFD antibodies that react with the central conserved domain of all six human Mcm proteins. Since MCM3p and MCM6p cannot be separated in the electrophoresis system used, the DEFD antibodies were removed from the membrane, which was then used for reprobing with MCM6-specific antibodies. C and D, immunoblotting with ORC2 and SAF-A antibodies, respectively. SAF-A is the scaffold attachment factor described by Göhring et al. (41).
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5), we may expect to see at least some ORC2p on Mcm-bearing nucleoprotein, if both proteins were present on one DNA fragment. This was not observed. Thus, the results of Fig. 6A support our conclusion that ORC2p and Mcm proteins are not located at identical or closely adjacent sites in HeLa cell chromatin. Based on the lengths of the DNA fragments in immunoprecipitated nucleoprotein (Fig. 6A), we estimate that the distance between bound ORC2p and bound Mcm proteins must be at least 1000 base pairs.

An inspection of Mcm-bearing nucleoprotein fragments is instructive (Fig. 6A). MCM3-specific antibodies precipitated nucleoprotein fragments with bound MCM3p, as expected, but these fragments also carried MCM5p and MCM7p. Likewise, MCM5-specific antibodies reacted with nucleoprotein carrying MCM5p as well as MCM3p and MCM7p. However, MCM4-specific antibodies precipitated nucleoprotein fragments with MCM4p, MCM6p, and MCM7p, but without MCM3p and MCM5p. MCM2p was present on immunoprecipitated nucleoproteins in amounts too low to be detected by the DEFD antibodies, but the protein could well be identified by MCM2-specific antibodies in all Mcm-bearing nucleoprotein (Fig. 6C).

These results are consistent with the sucrose gradient analysis of Fig. 3 where we detected stable MCM3p-MCM5p dimers (24) as well as cosedimenting MCM4p and MCM6p, while MCM7p overlaps the peaks of other Mcm proteins (Fig. 3). The results probably indicate a dynamic state of chromatin-bound Mcm proteins that are not necessarily organized as a hexameric complex such as soluble nucleosolic Mcm proteins (34, 44, 45).

The experiment shown in Fig. 6 was performed with nuclei from formaldehyde-treated cells arrested by a double thymidine block, but identical results were obtained with nuclei from cells treated with formaldehyde at 5 h and at 13 h after release from the block (not shown).

**DISCUSSION**

Evidence obtained through experiments in yeast and Xenopus had clearly shown that the replication preinitiation complex is assembled in an ordered sequence as Orc recruits CDC6p, which in turn recruits Mcm proteins (see Introduction). It is likely that the order of events is similar in mammalian cells. As a first step in the investigation of the preinitiation complex in human cells, we prepared chromatin from HeLa cells and digested it with micrococcal nuclease with the intention of producing chromatin fragments carrying the relevant protein components. We report now that, contrary to our expectation, Mcm proteins and ORC2p do not reside at closely adjacent sites in HeLa cells arrested by a double thymidine block at the G1/S phase boundary, as well as in cells released from the proliferation block.

Our conclusion is based on two different observations. First, chromatin was isolated and washed to effectively remove free nucleosolic Mcm proteins. Isolated chromatin was then treated with micrococcal nuclease to produce chromatin fragments, which were further investigated by gradient centrifugation. As shown above, gradient centrifugation separates digestion products with Mcm proteins from digestion products with ORC2p. After short digestion times ORC2p was found to be associated with faster sedimenting oligonucleosomes while most Mcm proteins were recovered in slower sedimenting parts of the gradients and were either free or bound to small pieces of DNA.

Second, chromatin proteins were covalently linked to DNA by a treatment of cells with formaldehyde. Cross-linked nucleoprotein fragments were obtained after equilibrium centrifugation in CsCl gradients and analyzed by immunoprecipitations using Mcm-specific or ORC2p-specific antibodies. Precipitated nucleoprotein fragments contained either Mcm
proteins or ORC2p, but not both. The DNA in immunoprecipitated nucleoprotein is 500–1000 base pairs long, which gives a lower limit for the distance between chromatin-bound ORC2p and chromatin-bound Mcm proteins. We shall perform experiments using cross-linked nucleoprotein complexes with increasingly longer DNA fragments to more precisely estimate the distance between Orc and Mcms in synchronously proliferating HeLa cells.

Several lines of evidence from studies with other systems suggest that Orc proteins and Mcm proteins are not necessarily bound to identical chromatid sites. Donovan et al. (11) were able to remove Orc proteins from yeast chromatin by salt extraction without affecting the binding of Mcm proteins; and Hua and Newport (14) could dissociate Xenopus Orc proteins, but not Mcm proteins in the presence of increased concentrations of Cdc2-cyclin A kinase. These data imply that Mcm proteins cannot be tethered to chromatin via Orc. This leads to a model suggesting that Orc and its associated CDC6p channels Mcm proteins to chromatin, but, once bound, Mcm proteins may move to other sites (12) which are closely adjacent in yeast (13), but may be more distant in mammalian cells. Furthermore, Aparicio et al. (17) could show that Mcm proteins leave their original sites after initiation of replication and move to DNA segments on both sides of the origin. It is now possible that the process of separation between Orc and Mcm proteins is already initiated in HeLa cells long before the beginning of DNA replication as measured by the incorporation of nucleoside precursors.

The experiments described in this communication also give some relevant new information with regard to the organization of chromatin-bound Mcm proteins. First, a short treatment with micrococcal nuclease suffices to release Mcm proteins from their chromatin sites. This may indicate that chromatin regions with bound Mcm proteins are relatively unprotected against micrococcal nuclease attack and therefore structurally distinct from bulk chromatin. The difference could be a more extended conformation of Mcm-carrying chromatin regions or a lack of nucleosomes in the vicinity of bound Mcm proteins. We are currently performing experiments to distinguish between these possibilities.

Second, centrifugation analysis of digested chromatin in low salt buffer shows that MCM3p appeared at two peak positions in the sucrose gradient, at 12 S together with MCM5p and at 4 S as a free monomer. On the other hand, MCM4p, MCM6p, and MCM7p form aggregates that are spread out over a larger section of the gradient (26). The result is consistent with the observations that a MCM3p-MCM5p dimer and a complex of MCM4p, MCM6p, and MCM7p, together with MCM2p, are obtained when HeLa chromatin is treated with 0.4 M NaCl for the preparation of isolated Mcm proteins (24, 46). The data are also in agreement with our cross-linking experiments which show that MCM3p-MCM5p complexes and MCM4p-MCM6p complexes tend to partition to distinct nucleoprotein fragments, but some overlap exists as MCM2p and MCM7p were found on all Mcm-bearing nucleoprotein fragments. These results suggest that the interactions between chromatin-bound Mcm proteins may be more dynamic and more complex than previously thought. Indeed, the composition of chromatin-bound Mcm complexes may vary with their functions before and during S phase. These functions have yet to be determined, although the recent report of Ishimi (46) on an Mcm-associated DNA-unwinding activity should be an interesting clue for further research.

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