Original Article

A Fraction of MCM 2 Proteins Remain Associated with Replication Foci During a Major Part of S Phase

(DNA replication / MCM proteins / immunofluorescence / replication foci / MCM paradox)

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Abstract. The essential role of MCM 2–7 proteins in the initiation of DNA replication in all eukaryotes is well known. Their role in replication elongation is supported by numerous studies, but there is still a knowledge gap in this respect. Even though biochemical studies have established an association of MCM proteins with replication forks, previous immunofluorescence studies in mammalian cells have suggested that MCM 2–7 proteins are displaced after replication initiation from sites of DNA replication. Therefore, we used a robust statistical method to more precisely analyse immunofluorescence localization of MCM 2 proteins with respect to the DNA replication foci. We show that despite the predominantly different localization of MCM 2 and replication signals, there is still a small but significant fraction of MCM 2 proteins that co-localize with DNA replication foci during most of S phase. The fluorescence localization of the MCM 2 proteins and DNA replication may thus reflect an active function of MCM 2 proteins associated with the replication foci and partially explain one facet of the "MCM paradox".

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Abbreviations: CCF – cross-correlation function, MCM – minichromosome maintenance, ORC – origin recognition complex.

Introduction

Minichromosome maintenance (MCM) 2–7 proteins were identified in a screen for *Saccharomyces cerevisiae* mutants with defects in minichromosome maintenance. Members of the MCM family belong to the AAA+ superfamily of ATPases and have been found in all eukaryotes. MCM proteins are defined by about 200 nucleotides long sequence, the MCM box, which includes two ATPase consensus motifs (for review see Forsburg, 2004; Bochman and Schwacha, 2009). Despite some variability in the results achieved, the present mainstream opinion is that the bulk of MCM 2–7 proteins is *in vivo* associated in a heterohexamer with 1 : 1 : 1 : 1 : 1 : 1 stoichiometry (Chong et al., 1995; Davey et al., 2003; see also Costa and Onesti, 2009; Remus et al., 2009). In nearly all species (with the exception of yeast), most of MCM 2–7 complexes have permanent nuclear localization, but their chromatin association is cell-cycle regulated (Forsburg, 2004; Bochman and Schwacha, 2009). The recruitment of the MCM 2–7 complexes to chromatin starts in late mitosis and continues until the end of G1 phase, with the number of chromatin-associated MCM 2–7 complexes at the end of G1 phase in various organisms exceeding the number of replication origins/origin recognition complexes (ORCs) by a factor of 10 to 100 (Krude et al., 1996; Dimitrova et al., 1999; Prasanth et al., 2004; Takahashi et al,. 2005). Chromatin loading of these complexes is mediated by CDC6 and CDT1 proteins, which interact with the ORC proteins to form a pre-replication complex at "specific" replication origins (reviewed in Blow and Ge, 2008; Gilbert, 2010; Méchali, 2010). Before DNA polymerases can begin DNA replication from specific origins, a fraction of loaded MCM 2–7 complexes have to be activated by two kinases, CDC7/DBF4 and S-CDKs (Bell and Dutta, 2002). This process involves gradual assembly of Cdc45, GINS, Sld2, Sld3 and Dpb11 proteins/protein complexes. Cdc45 and GINS proteins trig-

ger DNA unwinding and work together with MCM proteins (known as CMG complex) like DNA helicase (Gambus et al., 2006; Moyer et al., 2006; Costa and Onesti, 2009; Ilves et al., 2010). MCM 2–7 complexes dissociate from chromatin as DNA replication proceeds and their re-association is prevented by CDKs and geminin until late mitosis (McGarry and Kirschner, 1998; Nguyen et al., 2001). This mechanism ensures that DNA is replicated only once during S phase.

The above-described process of MCM 2–7 loading and activation is common to all eukaryotes, but there is a certain knowledge gap about the role of MCM 2–7 during DNA replication. *In vitro* studies demonstrate helicase activity of the CMG complex (Gambus et al., 2006; Moyer et al., 2006; Ilves et al., 2010) and also association of the CMG complex with the replication fork (Calzada et al., 2005; Pacek et al., 2006). However, immunofluorescence studies in mammalian cells have indicated that MCM 2–7 proteins are displaced from the sites of DNA replication and are present only on unreplicated chromatin. The failure to demonstrate the association of MCM proteins with replication foci, together with observations that the number of chromatin-bound MCM 2–7 complexes at the end of G1 phase largely exceeds the number of replication origins/ORC complexes, is known as "the MCM paradox" (Dimitrova et al., 1999; Edwards et al., 2002; Hyrien et al., 2003; Takahashi et al., 2005).

To expand our knowledge with respect to the first facet of the "MCM paradox", we used a robust statistical method to more precisely analyse immunofluorescence localization of MCM 2 proteins with respect to replication foci. We were able to show that despite the predominant different localization of these MCM proteins and replication signal, there was a small but significant fraction of MCM 2 proteins that were, within the resolution limit of light microscopy, associated with replication foci during a major part of S phase.

Material and Methods

Cell culture, replication labelling, extraction and fixation

Human HeLa cells were grown on circular coverslips and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (Sigma-Aldrich, Prague, Czech Republic), 1% glutamine, 1% penicillin, 1% streptomycin and 0.85 g/liter $NaHCO_3$ at 37 °C in a humidified atmosphere containing 5% CO_2. Sites of DNA replication were labelled by 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen, Paisley, UK) that was added directly to the medium in a final concentration of 10 μM for 20 min. Then the cells were either briefly washed in PBS, fixed by 2% formaldehyde in PBS for 30 min and permeabilized by 0.2% Triton X-100 in PBS for 5 min or extracted before fixation. Extensive assays were performed concerning the latter procedure and finally the approach of Martini et al. (1998) was chosen with following modifications. Cells were extracted only with 0.1% Triton X-100 in CSK buffer (10 mM Pipes-KOH, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$) at room temperature for 5 min. Then the extraction solution was carefully replaced by 2% formaldehyde in CSK buffer for 30 min.

Fluorescence labelling and confocal microscopy

MCM 2 was detected using mouse anti-BM28 (BD Transduction Laboratories; Oxford, UK; Todorov et al., 1995) and simultaneously either H4Ac was detected using polyclonal rabbit anti-acetyl-histone H4 (Millipore, Prague, Czech Republic), or MCM 3 or MCM 7 proteins were detected by the corresponding affinity-purified polyclonal rabbit antibodies (Burkhart et al., 1995; Schulte et al., 1996) kindly provided by Rolf Knippers. This was followed by incubation with secondary Cy3-conjugated anti-mouse (Jackson ImmunoResearch Laboratories, Suffolk, UK) and Alexa488-conjugated anti-rabbit (Invitrogen, Molecular Probes, Paisley, UK) goat antibodies. Consequently, EdU was detected with Click-iT Alexa647 EdU Imaging Kit (Invitrogen, Molecular Probes), DNA with DAPI, and the samples were embedded in mowiol. For negative controls, an omission of primary antibodies was performed. In the case of MCM 2 proteins, an isotype IgG1 antibody, 18437 (Abcam, Cambridge, UK), was also used. Mid confocal sections were acquired using a confocal Leica SP5 microscope (Leica Microsystems GmbH, Wetzlar, Germany) with 63x PlanApochromat/1.4 NA oil immersion objective. Individual fluorescence signals were always detected by sequential excitation using 405 nm Diode, 488 nm Argon, 561 nm DPSS and 633 nm HeNe lasers and with carefully set up emission bandwidths to avoid possible cross-talks.

Chase experiment

After 10 min pulse of EdU, the cells were incubated in fresh medium without EdU for 90 min and processed identically as described above.

Image analysis

The cross-correlation function (CCF = CCF(Δx)) (Steensel et al., 1996) was calculated as a Pearson's correlation coefficient: cov(R(Δx),G)/(var(R(Δx)).var(G)), where R and G represent sets of red and green pixel intensities in the image, while variable horizontal shift of Δx pixels is applied to the red channel. The pixel size was 60 nm. CCF calculation was done by our software created in Matlab (The MathWorks, Natick, MA). This software with user instructions can be freely downloaded from our website: http://lge.lf1.cuni.cz/esoftware.html.

Correlation coefficient values range from -1 to 1. We emphasize that the shapes of CCF curves may be complicated and in such a case, the interpretation of CCF(Δx) requires a careful analysis. A straightforward but simplified explanation of the established CCF curves is that a higher positive peak at Δx = 0 indicates a significant non-random overlap/co-localization, values evenly dis-

tributed around zero reflect random mutual distribution, and a negative peak indicates anti-co-localization/"repulsion" of the two investigated fluorescence signals.

Results

We tested anti-MCM 2, 3 and 7 antibodies for specificity in detection of the MCM protein distribution *in situ* by immunofluorescence staining. We had at our disposal only small aliquots of anti-MCM 3 and 7 affinity-purified rabbit antibodies and thus were not able to perform a complete list of experiments. In order to cover the whole range of experiments, we focused on the use of the anti-MCM 2 mouse monoclonal antibody that gave the characteristic immunofluorescence staining and was commercially available.

In fixed and immunolabelled cells, the MCM 2 proteins distributed in the form of fluorescence foci homogenously scattered (except nucleoli) over the nucleus (Fig. 1 and results not shown). In order to visualize only proteins tightly bound to chromatin, we used the procedure of mild Triton-X-100 extraction prior to fixation (for details see Material and Methods) that is commonly used in the chromatin field (Krude et al., 1996; Martini et al., 1998; Dimitrova et al., 1999; Ekholm-Reed et al., 2004; Prasanth et al., 2004). The extraction procedure removed most soluble nuclear proteins while retaining the chromatin-bound proteins, but without the use of high sucrose concentration it led to dramatic changes of the nuclear shape. The use of 300 mM sucrose also had some effect on nuclear structures. The most pronounced effect consisted in chromatin condensation, and thus locally increased signal, as visualized in confocal optical sections of DAPI staining and acetylated histone H4 (H4Ac) immunodetection (Fig. 1) (Albiez et al., 2006; Richter et al., 2007). These two markers were further used as convenient labelling controls for chromatin/DNA and euchromatin, which is characterized by a high level of histone acetylation (Bartova et al., 2008). It is important to realize that chromatin condensation also influenced MCM 2 protein distribution after extraction (Fig. 1). The results obtained with anti-MCM 3 and 7 antibodies were compatible with results of the MCM 2 proteins (results not shown). In summary, we found the above-described procedure as the best deal between overall preservation of nuclear structure and removal of soluble nuclear proteins.

We then analysed the relative binding sites of anti-MCM 3 and 7 antibodies with respect to those of the MCM 2 proteins. We found high co-localization of MCM 2 with MCM 7 (Fig. 2) and MCM 3 (data not shown) as documented by the analysis of the cross-correlation function (CCF; for an explanation of the CCF curves see Material and Methods; see also Steensel et al., 1996 and Masata et al., 2005). This method represents a robust statistical tool that allows us to study relative distributions of two fluorescence signals. Since maximal CCF values do not usually exceed 0.8 (see Masata et al., 2005 for the explanation), our results suggest that MCM 2 and 3 proteins as well as MCM 2 and 7 proteins are in a complex. Even though we cannot claim that the three MCM proteins are in a complex, our results are compatible with the results of previous studies showing that the bulk of bound MCMs were *in vivo* in

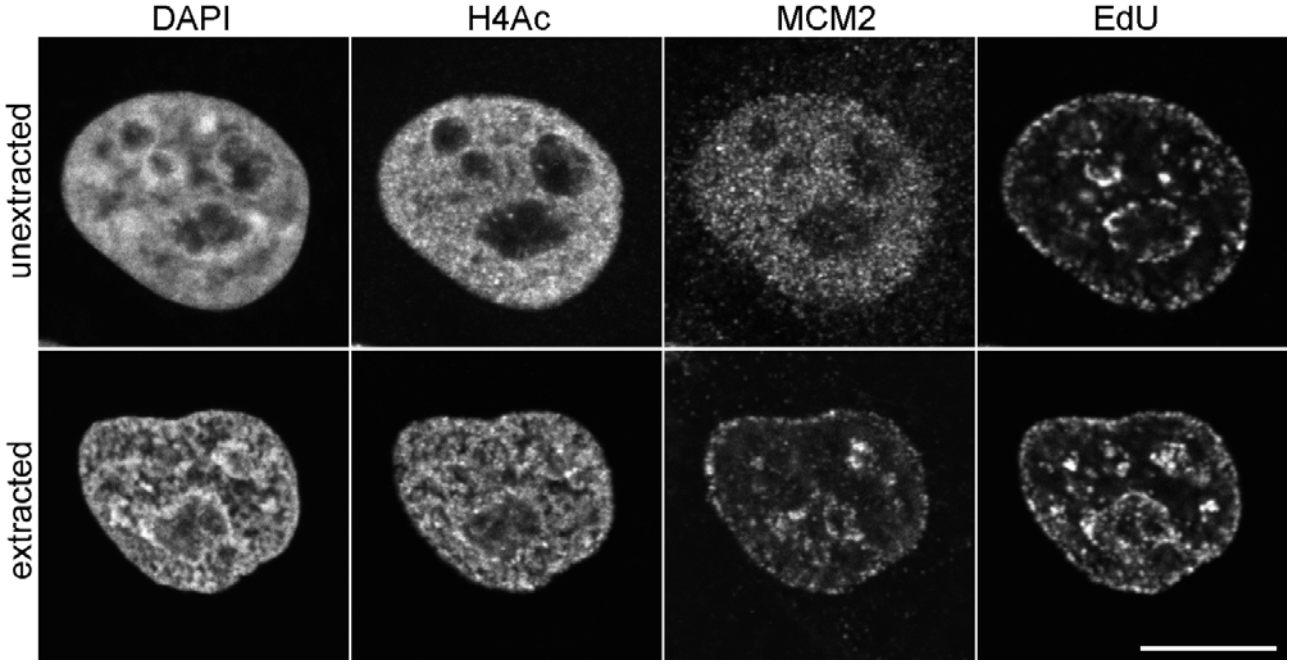

Fig. 1. Influence of extraction on nuclear architecture
The organization of unextracted (first row) and extracted (second row) cell nucleus in a mid confocal optical section is documented by DNA (DAPI), euchromatin (H4Ac), MCM2 and DNA replication (EdU pulse) staining/labelling. Chromatin condensation due to the extraction procedure is clearly visible, but the most striking effect is the removal of soluble MCMs. This allows us to analyse the localization of chromatin-bound MCMs. Bar: 10 μm.

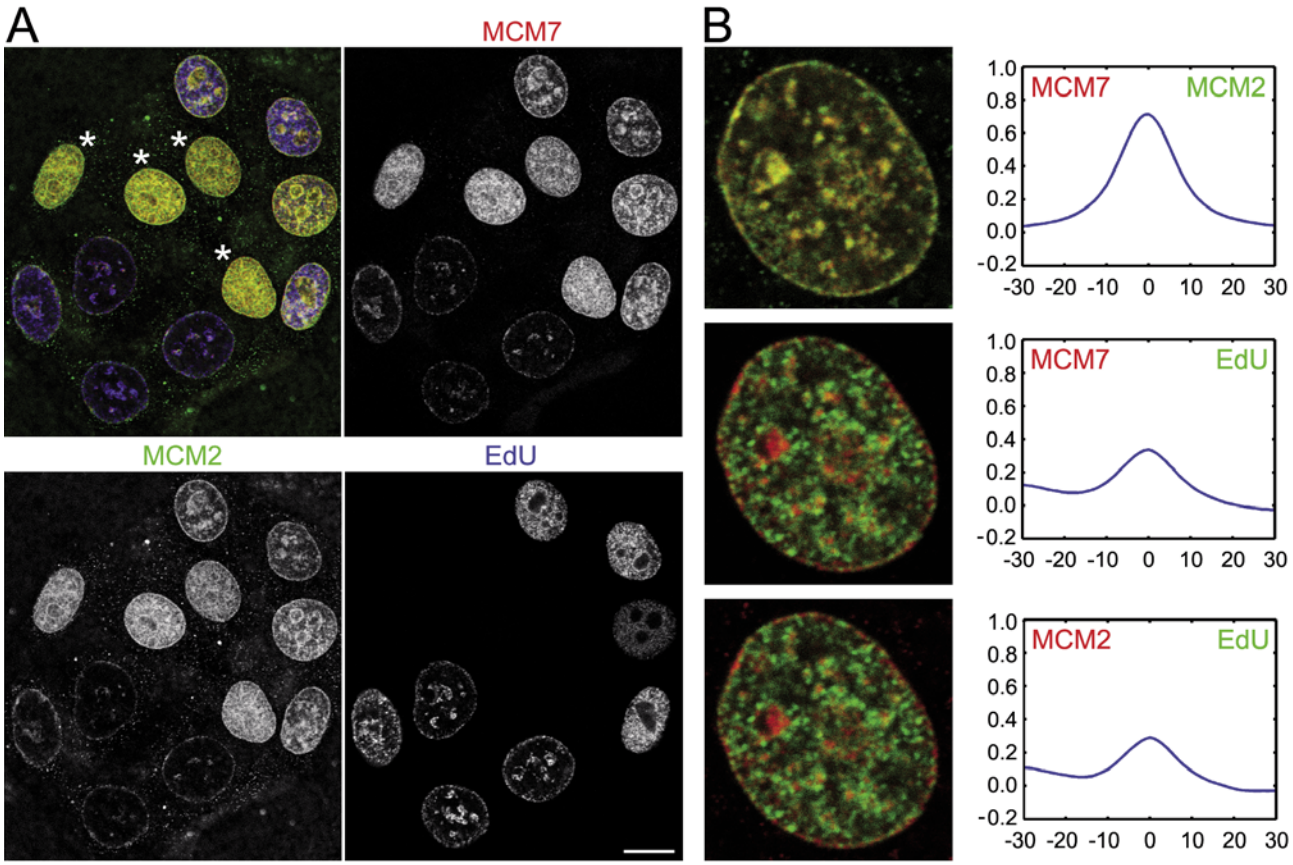

Fig. 2. Co-localization of different MCM proteins in extracted cells during the cell cycle
A: High degree of co-localization between MCM 7 (red channel in merge image) and MCM 2 (green channel) in nuclei of S-phase cells (as documented by a positive EdU signal in blue channel) and in G1-phase nuclei (nuclei marked by white asterisks) are shown in a confocal section. The comparison of MCMs and EdU signals in all relevant nuclei indicates that the replication signal follows the MCM signal. Bar: 10 μm.
B: High degree of co-localization between MCM 7 and MCM 2 is confirmed by CCF on individual nucleus (the first row). Similarity of MCM 7 and MCM 2 signals is also indicated by analogous CCF curves with respect to EdU (the second and the third rows).

the form of heterohexameric complexes of MCM 2–7 (Chong et al., 1995; Davey et al., 2003).

We also detected DNA synthesis to determine the S-phase cells and corresponding MCM patterns (Figs. 1, 2). We labelled S-phase cells by a 20 min long pulse with the nucleotide analogue 5-ethynyl-2'-deoxyuridine (EdU) prior to extraction and fixation (Salic and Mitchison, 2008). A comparison of DNA replication and MCM patterns indicated that MCM 2 and 7 proteins, as well as MCM 3 proteins (data not shown), were located on chromatin the DNA of which was to be replicated and were gradually displaced from chromatin along with the progression of S phase. Our observations were thus in harmony with the findings reported previously (Krude et al., 1996; Prasanth et al., 2004).

To analyse changes in the MCM 2 pattern in detail, we divided the extracted cells according to their replication signal into five temporarily ordered groups: G1 – no replication signal but intense MCM 2 staining, VE – very early S that is characterized by lower number of replication foci located in the nuclear interior, E – early S composed of similarly sized foci homogenously scattered in the nucleus (except nucleoli), EM – early to mid S when some foci start to form "chains" mainly along the nuclear envelope and nucleoli, ML – mid to late S that is marked by the presence of larger foci; however, we excluded from the analysis the cells in the (very) late S since the MCM 2 signal was either too weak or non-detectable. A representative nucleus for each group is shown in Fig. 3. While DAPI and H4Ac signals did not change extensively with the progression of interphase, a gradual disappearance of MCM 2 signal was clear. Mutual relations of MCM 2 proteins with DNA replication, chromatin/DNA, or euchromatin are shown in the respective merge images. A significant overlap with the replication signal was seen only in later periods of S phase that were characterized by the presence of large replication foci. These, however, consisted of clustered smaller foci that were not resolved by light microscopy (Raska et al., 1989, 1991; Leonhardt et al., 2000; Koberna et al., 2005). Overlap of MCM 2 proteins with euchromatin was high at the border of G1/S phase and then quickly fell. This well reflected observations that euchromatin regions generally replicate earlier than the hetero-

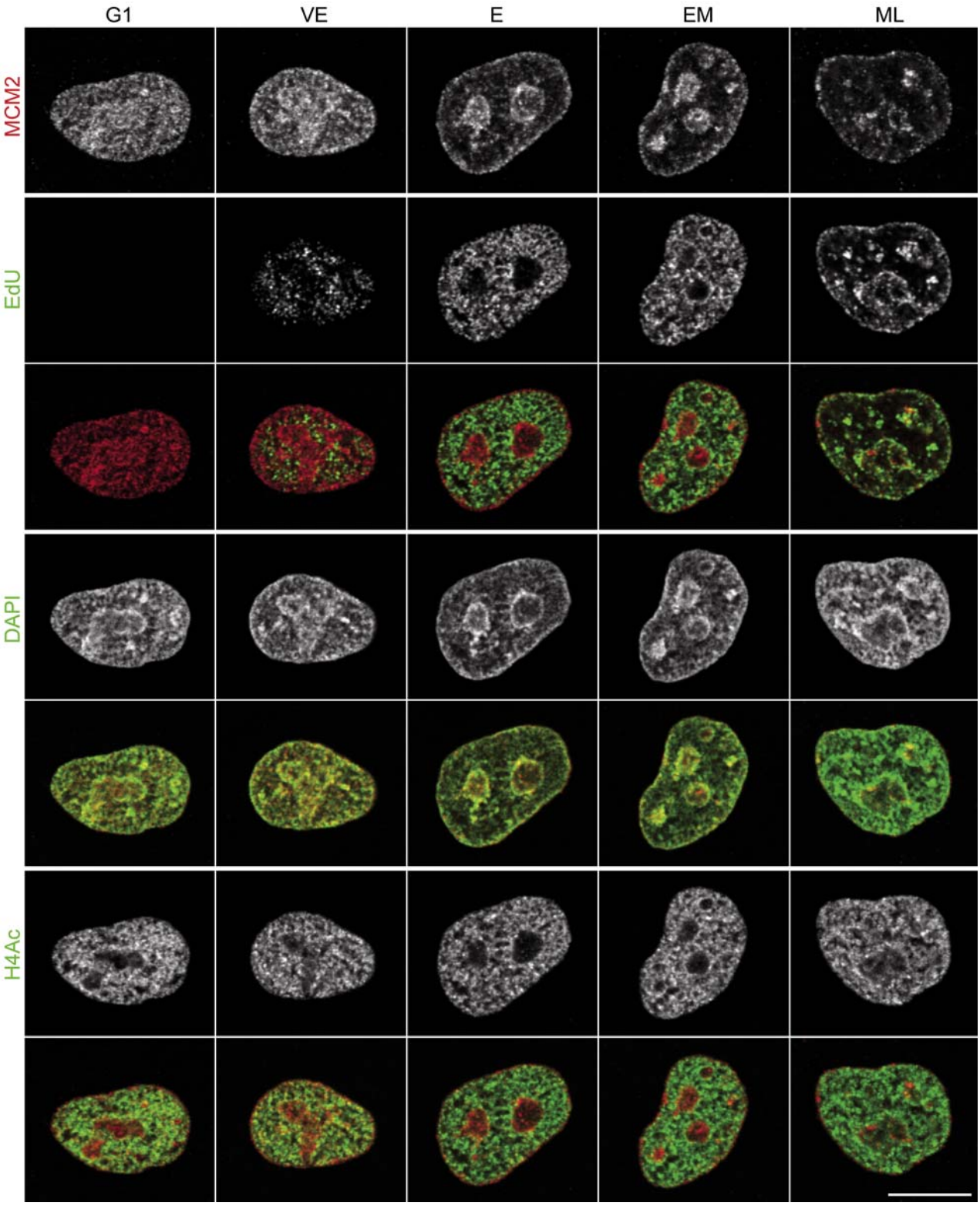

Fig. 3. Dynamics of chromatin-bound MCMs from late G1 until mid to late S phase period of the cell cycle
Extracted cells were divided according to the patterns of the replication signal into five groups: late **G1** – no replication signal and intense MCM 2 signal, **VE** – very early S, **E –** early S, **EM** – early to mid S, and **ML –** mid to late S. Representative MCM patterns for each group are shown in the first row and in merge images (red channel). EdU, DAPI and H4Ac signals of same confocal sections are presented on marked rows and in merge images (green channel one row below). While DAPI and H4Ac distributions are relatively stable, MCM2 and EdU patterns are highly variable during the cell cycle. In merge images, a partial overlap between MCM2 and EdU is visible only in mid to late S phase and between MCM2 and H4Ac in G1 to early S phase. On the other hand, MCM2 and DAPI co-localize in all shown images. However, the degree of co-localization decreased concomitantly with the gradual disappearance of chromatin-bound MCM 2 as the S phase proceeded. Bar: 10 μm.

chromatin ones (Gilbert, 2002). Clear changes were also apparent between MCM 2 and DAPI signals, showing the gradual decrease of the chromatin-bound fraction of MCM 2 proteins during the S phase. Results with MCM 3 and 7 proteins were compatible with those obtained for MCM 2 (data not shown). The visual analysis was thus basically in agreement with previous immunocytochemical studies claiming the gradual displacement of MCM proteins from chromatin along with the progression of S phase (Krude et al., 1996; Prasanth et al., 2004).

To establish possible finer regularities that may not be obvious from images by visual inspection, we implemented again the CCF analysis for the evaluation of signals (Steensel et al., 1996; Masata et al., 2005). Results of the CCF analysis are shown in Fig. 4, and we bring attention of the reader to the behaviour of DAPI/H4Ac curves that serve well for understanding the analysis performed. In this case, CCF curves between MCM 2 and DAPI/H4Ac confirmed visual assessments. However, a surprising effect was seen in CCF curves between MCM 2 and EdU. From the visual analysis alone we expected a negative peak for MCM 2 versus EdU in early S, similarly as it was for MCM 2 versus H4Ac in mid to late S (black CCF curve) that testified for a "repulsion" of signals. However, there were small but distinct "positive" peaks (white arrows in Fig. 4) in the centres of negative values for very early and early S phase. Such a character of the curves meant that, in spite of a predominantly different localization of both signals, there was – at the resolution limit of light microscopy – still a small but significant fraction of MCM 2 proteins which were associated with DNA replication foci. Importantly in this respect, negative controls (omission of primary antibodies or use of isotype control antibodies) did not show such distinct peaks (data not shown). Moreover, the presence of such a positive peak (Fig. 4) was also obvious for early to mid S phase, but was partially masked in mid to late S. This masking resulted from the fact that the MCM 2 and EdU signals were in close proximities due to an appearance of larger heterochromatin structures that were typically replicated in later S and encompassed multiple clustered replication foci (Raska et al., 1989, 1991; Leonhardt et al., 2000; Koberna et al., 2005). Due to the exhaustion of our stock of anti-MCM 3 and 7 antibodies, we were unable to perform the same thorough analysis for MCM 3 and 7 proteins. However, the limited set of performed experiments indicated that the CCF analysis for MCM 3 or 7 versus EdU signal could provide compatible results (data not shown and graphs in Fig. 2) with those obtained with the MCM 2 proteins. In summary, our results thus indicated that a small but significant fraction of MCM 2 proteins were associated with DNA replication foci through a major part of S phase. The inability to demonstrate the MCM signal associated with replication foci in the previous studies was probably due to a sharp drop in the number of immunofluorescence MCM targets associated with replication foci during the progression of S phase.

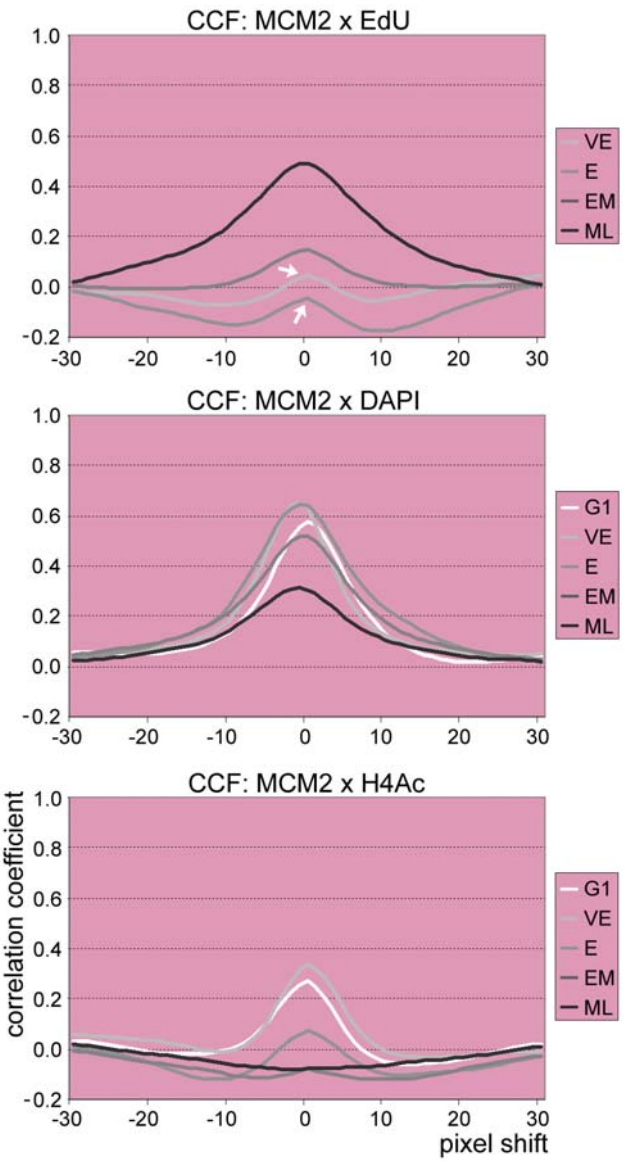

Fig. 4. Quantitative analysis of chromatin-bound MCM 2 with respect to DNA synthesis and chromatin
CCF curves between MCM 2 and EdU/ DAPI/H4Ac signals are shown in the corresponding graphs. White to black curves of different greyness represent specific periods of the cell cycle as described in the text and indicated in legends next to the graphs. Most of the curves fit visual assessments from Fig. 3. However, two small "positive" peaks marked by white arrows are seen. These peaks testify for the existence of an MCM fraction that co-localizes with replication foci even in early S phase. Presented CCF curves were calculated as the average of five images for each group in Figs. 4 and 5.

In order to find out whether the above-described fraction of MCM 2 proteins associated only with active replication foci, we performed 90 min chase experiment for the replication signal generated by the 10 min EdU pulse labelling in early S-phase cells (Fig. 5). At this period of the cell cycle, replication foci are, in contrast to mid and late S-phase foci, spatially well separated (Raska et al., 1989, 1991; Koberna et al., 2005). After the chase, the

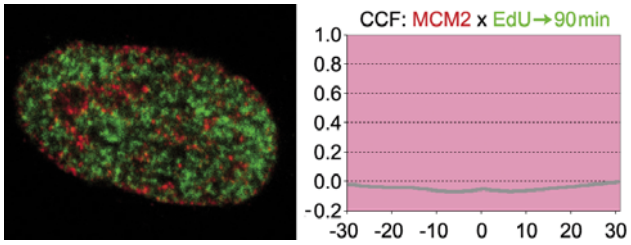

Fig. 5. Quantitative analysis of chromatin-bound MCM 2 with respect to DNA replication signal after chase
The cells were, after 10 min incorporation of EdU, cultured for 90 min in medium free of EdU and processed for immunocytochemistry as in Fig. 4 for EdU and MCM 2 signals. In the graph, the CCF curve between MCM2 and EdU signals is shown for cells that incorporated EdU in the early S phase. With respect to Fig. 4, note that the small "positive" peak has almost disappeared. This indicates that the remaining fraction of MCM proteins disassociated from former replication sites.

distinct "positive" peak of CCF curve MCM 2 versus EdU basically disappeared. This indicated that the remaining MCM proteins disassociated from these sites when replication had been finished. Compatible pulse chase results were also obtained with MCM 3 and 7 proteins (data not shown).

Discussion

Despite enormous progress in the understanding of molecular mechanisms standing behind the DNA replication process accomplished in the last 10 years (Bochman and Schwacha, 2009; Costa and Onesti, 2009; Méchali, 2010), a synthetic view how the DNA replication proceeds in space and time in the living cell, with an immense number of various factors involved, is still missing. The replication process in eukaryotes is not only a complex one, but also experimentally difficult to tackle. In this study, we focused on a small gap in the knowledge within the frame of the replication process – the observation of failures to demonstrate by immunofluorescence an association of the MCM proteins with the replication foci as documented in previous studies (Dimitrova et al., 1999; Edwards et al., 2002; Hyrien et al., 2003; see also Takahashi et al., 2005).

The fluorescence localization of a fraction of MCM 2 proteins associated with replication foci as shown in this study is consistent with the fraction of MCM 2 proteins involved at a given time in the replication process. Our results may thus partially explain the first facet of the "MCM paradox". At the same time, our results do not allow us to comment on any specific function of these proteins. Along these lines, we are unable to comment on the second facet of the "MCM paradox" – the observation that the number of chromatin-bound MCM 2–7 complexes at the end of G1 phase largely exceeds the number of replication origins/ORC complexes.

The MCM proteins are said to be involved in the DNA unwinding preceding the DNA replication process (Bochman and Schwacha, 2009; Costa and Onesti, 2009). There are possibly two most extreme models that describe the distribution of MCM proteins/complexes with respect to replication sites. They either propose an association of MCM proteins with the replication forks or localize them at distance from the forks (Aparicio et al., 1997; Ritzi et al., 1998; Claycomb et al., 2002; Laskey and Madine, 2003; Calzada et al., 2005; Pacek et al., 2006). We indeed mapped the MCM 2 protein with respect to replication foci in this study, but we were limited by the imposed low resolution of the microscope being 200 nm. The diameter of most replication foci seen in the electron microscope is well over 100 nanometers (Raska et al., 1989; Koberna et al., 2005) but, at the same time, lower that the resolution limit of the fluorescence microscope. Importantly in this respect, the apparent size of fluorescing replication foci was about 400 to 500 nm and the length of the stretched DNA segment of 1000 bp in B configuration is only about 340 nm. Our data are thus compatible with the two models, but do not allow us to distinguish whether the fraction of MCM 2 proteins associate with replication forks or are located at a distance not exceeding roughly 200 nm, the structure of replication foci being not known anyway.

In summary, the presence of a small fraction of MCM 2 proteins associated with replication foci during most of S phase as established in this study may explain, at least partially, the first facet of the "MCM paradox" as best illustrated by Dimitrova et al. (1999).

Acknowledgments

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