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Human replication proteins hCdc21, hCdc46 and P1Mcm3 bind chromatin uniformly before S-phase and are displaced locally during DNA replication

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SUMMARY

Members of the Mcm-protein family have recently been shown to be involved in restricting DNA replication to a single cycle in *Xenopus laevis* egg extracts. In this study, we extended these observations to human somatic cells and analysed the localisation of the human Mcm-proteins Cdc21, Cdc46 and P1Mcm3 in replicating HeLa cell nuclei. These Mcm-proteins are entirely nuclear in interphase cells and apparently exist in two populations: a nucleosolic population, and a population bound to a nuclear structure, most likely chromatin. The bound population is detected throughout the nucleus in late G_1 and early S, and at discrete subnuclear sites following further progression of S-phase. We use high resolution confocal microscopy to determine the subnuclear sites of chromatin-bound Mcm proteins in comparison to the sites of replicating DNA. Importantly, hCdc21, hCdc46 and P1Mcm3 do not co-localise with replication foci, instead these proteins appear to coincide with subnuclear sites of unreplicated chromatin. During progression of S-phase hCdc21, hCdc46 and P1Mcm3 are displaced from their site on chromatin at the time when this site is replicated. Consequently, early replicating sites do not contain bound hCdc21, hCdc46 or P1Mcm3 during later stages of S-phase. Furthermore, G_2 nuclei and condensed chromatin in mitotic cells do not contain bound hCdc21, hCdc46 or P1Mcm3.

Thus, the human Mcm-proteins Cdc21, Cdc46 and P1Mcm3 are not concentrated at sites of DNA replication. Instead, they appear to be present only on unreplicated chromatin and are displaced from replicating chromatin, consistent with a role in monitoring unreplicated chromatin and ensuring only a single round of DNA replication per cell cycle.

Key words: Cell division cycle, S phase, Replication focus, Mcm-protein, hCdc21, hCdc46, P1Mcm3, Licensing factor

INTRODUCTION

The replication of the eukaryotic genome is highly regulated in a temporal fashion. Although individual replicons are initiated at different times throughout S-phase (Hand, 1978) the genomic DNA within a nucleus is replicated only once and re-replication is prevented. A block preventing intact G_2 nuclei from re-replicating was discovered in cell fusion experiments (Rao and Johnson, 1970). This regulation was further analysed using egg extracts from *Xenopus laevis*. All DNA within a G_1 nucleus added to the extract is replicated precisely once and re-replication occurs only after nuclei are allowed to pass mitosis or after the nuclear membrane is permeabilised (Blow and Laskey, 1986, 1988; Leno et al., 1992). Therefore, the chromatin of postreplicative nuclei must be modified in a way that prevents re-replication. Blow and Laskey (1988) proposed a 'licensing factor' model of replication control that could explain these observations. An essential replication licensing factor would bind to chromatin before S-phase and would be inactivated during DNA replication. The hypothetical factor cannot cross the nuclear envelope and would therefore be excluded from the nucleus until mitosis. New licensing factor would be able to enter the nucleus after nuclear membrane breakdown at mitosis allowing initiation to occur in a once per cell cycle fashion.

In the yeast *Saccharomyces cerevisiae* genetic analyses have defined a class of mutants involved in the initiation of DNA replication (Tye, 1994). These mutants are unable to sustain the replication of minichromosomes bearing autonomously replicating sequences as origins of DNA replication and were therefore designated mcm-mutants (mini-chromosome maintenance). The *Saccharomyces cerevisiae* mutants *mcm2*, *mcm3*, *mcm5* (alias *cdc46*) as well as the *Schizosaccharomyces pombe* mutant *cdc21* have similar phenotypes (Hennessy et al., 1990, 1991; Yan et al., 1991, 1993; Chen et al., 1992; Coxon et al., 1992). Furthermore, the affected genes code for highly homologous proteins. The Mcm2, Mcm3 and Mcm5/Cdc46 proteins in yeast have attracted particular interest, as they enter the nucleus in late mitosis, disappear from the nucleus during DNA replication and reappear during the following mitosis

(Hennessy et al., 1990; Yan et al., 1993). This behaviour is reminiscent of that postulated for licensing factor, although in yeast the nuclear membrane does not break down during mitosis.

Proteins homologous to yeast Mcm3 have been identified in humans (alias P1; Thömmes et al., 1992), mouse (Kimura et al., 1994; Starborg et al., 1995), *Xenopus laevis* (Madine et al., 1995a; Kubota et al., 1995) and other species. The human counterparts of Mcm2, Mcm5/Cdc46 and Cdc21 and of further members of the Mcm-protein family have recently been identified and cloned (Hu et al., 1993; Todorov et al., 1994; Burkhart et al., 1995; Schulte et al., 1995; Musahl et al., 1995). In human cells these proteins apparently form complexes that can be immunoprecipitated: P1Mcm3 is complexed with hCdc46/Mcm5 (Burkhart et al., 1995) and hCdc21 is complexed with two other Mcm-proteins, p85 and p105. Furthermore, the Mcm2-homologue, protein BM28, seems to be loosely attached to this latter complex (Musahl et al., 1995).

Recent evidence indicates that the *Xenopus* homologue of Mcm3 (XMcm3) is in fact a part of the replication licensing activity (Madine et al., 1995a,b; Kubota et al., 1995; Chong et al., 1995). Immunodepletion of XMcm3 also removes XMcm2 and XMcm5 from the *Xenopus* egg extract. In the depleted extract, permeabilised G_2 nuclei and *Xenopus* sperm nuclei fail to replicate, whereas G_1 nuclei, containing endogenous Mcm3-protein still replicate (Madine et al., 1995a,b). In untreated extracts, XMcm3-immunostaining increases in naive sperm nuclei prior to replication and decreases during replication (Madine et al., 1995a,b; Kubota et al., 1995; Chong et al., 1995). Similar changes of immunofluorescence patterns have been also described for murine Mcm3 (Kimura et al., 1994; Starborg et al., 1995) and for BM28 (Todorov et al., 1995), consistent with an involvement of the Mcm proteins in DNA replication in somatic vertebrate cells. It is therefore of interest to analyse the intranuclear localisation of these Mcm-proteins in comparison to the subnuclear sites of DNA replication. This analysis will allow discrimination between an involvement of these Mcm-proteins either as replication factors engaged in the elongation of DNA replication, or as 'licensing proteins' involved in distinguishing unreplicated from replicated chromatin.

In the nucleus DNA replication occurs at discrete sites termed replication foci (Nakamura et al., 1986; Mills et al., 1989; Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Kill et al., 1991; Cox and Laskey, 1991; Fox et al., 1991; O'Keefe et al., 1992; Hassan and Cook, 1993; Hozák et al., 1993, 1994). Replication foci can be visualised by immunofluorescence microscopy using probes directed against biotinylated dUTP or bromodeoxyuridine (BrdU) incorporated into pulse-labelled nascent DNA in situ (Nakamura et al., 1986; Mills et al., 1989; Nakayasu and Berezney, 1989). During progression of S-phase in somatic mammalian cells, the pattern of replication foci changes in a characteristic way (Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Fox et al., 1991; Hassan and Cook, 1993). Replication starts in the euchromatin of the nucleus at approximately 100-300 foci that initially spare the nucleoli and heterochromatin at the nuclear periphery. In mid S-phase these foci are located near the perinuclear heterochromatin and surround the nucleoli. In late S phase, only a few intense and larger replication foci are located at the nuclear periphery and within the nucleoli. Proteins involved in the elongation steps of cellular DNA replication have been shown to co-localise with these discrete replication foci in a variety of cell types (Bravo and Macdonald-Bravo, 1987; Hutchison and Kill, 1989; Adachi and Laemmli, 1992, 1994; Leonhardt et al., 1992; Cardoso et al., 1993; Krude, 1995).

This paper analyses the subnuclear localisation of the human Mcm-proteins hCdc21, hCdc46 and P1Mcm3 in relation to the discrete sites of DNA replication during S-phase. These Mcm proteins associate with chromatin throughout the nucleus before the onset of DNA replication. They do not co-localise with replication foci during S-phase. Importantly, they are displaced from their sites on chromatin during replication and are therefore not bound to replicated chromatin in late S, G_2 and mitosis, consistent with a role in monitoring unreplicated chromatin.

MATERIAL AND METHODS

Antibodies

The hCdc21 and hCdc46 proteins were expressed in bacteria using the pRSET expression system (Invitrogen) and partial human cDNA clones (Musahl et al., 1995; Burkhart et al., 1995). These proteins were used to raise antibodies in rabbits as described (Hu et al., 1993). Monospecific antibodies were obtained by affinity purification. The antigen was coupled to tresyl-activated agarose (Nalgene) as specified by the supplier. Antiserum was added, washed extensively and eluted as detailed previously (Schulte et al., 1995).

Cell culture

HeLa-S3 cells were cultured as exponentially growing subconfluent monolayers on 145 mm plates in DME-medium (Gibco), supplemented with 5% foetal calf serum (Gibco), 10 i.u./ml penicillin (Sigma) and 0.1 mg/ml streptomycin (Sigma).

For cell cycle synchronisations (Johnson et al., 1993), cells were first arrested in early S phase by two subsequent excess thymidine blocks (2.5 mM thymidine (Sigma) in the culture medium for 25 hours, seperated by a 12 hour interval without thymidine). A population of cells predominantly in the G_2 phase of the cell cycle was obtained by releasing the arrested early S population in culture medium for 7 hours. A population of cells in the G_1 phase was obained by releasing blocked early S-phase cells in culture medium for 3 hours, followed by a block in mitosis by adding 20 ng/ml nocodazole (Sigma) to the culture medium for an additional 10 hours. These mitotic cells were then released into fresh culture medium for 8 hours. Flow cytometry and immunofluorescence microscopy showed that this G_1 preparation contained 95% of nuclei in G_1 and 5% in S, and the G_2 preparation contained 55% of nuclei in G_2 and 45% in late S (data not shown). During the preparation of nuclei for immunofluorescence microscopy and flow cytometry the condensed chromatin from mitotic cells was lost (see below, Fig. 3) and is therefore excluded from this analysis.

Preparation of permeabilised HeLa cells

A suspension of permeabilised HeLa cells was prepared essentially according to the method of Heintz and Stillman (1989) with minor modifications (Krude, 1995). The cells were washed twice with ice-cold hypotonic buffer: 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, potassium salt (K-Hepes, pH 7.8), 5 mM potassium acetate, 0.5 mM $MgCl_2$, 0.5 mM DTT. All subsequent steps were carried out at 4°C. The cells were allowed to swell for 10 minutes in 20 ml hypotonic buffer per plate, and all excess buffer was removed. The cells were scraped off the plates and disrupted with 25 strokes in a dounce homogeniser (Wheaton), using pestle B. The integrity of the nuclei in the suspension was checked by light microscopy.

Extraction procedures of isolated nuclei

The nuclear suspension was centrifuged at 4,000 rpm for 2 minutes in an Eppendorf 5415C centrifuge. For a hypotonic extraction, the pelleted nuclei were washed, resuspended and incubated for 1 hour on ice in hypotonic buffer. The nuclei were again pelleted, washed and finally resuspended in 500 μl PBS. For a preparation of Triton-extracted nuclei, the pelleted nuclei were washed, resuspended and extracted for 10 minutes on ice in PBS containing 0.1% Triton X-100 (Kimura et al., 1994). The nuclei were then pelleted, washed and finally resuspended in 500 μl PBS. For a preparation of DNase I-treated nuclei, the pelleted nuclei were resuspended in 400 μl hypotonic buffer containing 2,000 U pancreatic DNase I (Sigma). Nuclei were incubated for 1 hour at 37°C, pelleted and then extensively washed in hypotonic buffer. The extent of the DNase I digestion was controlled by the release of oligonucleotides (determined by A_{260}) from the digested nuclei and by staining the residual nuclear structure with Hoechst 33258 (see Fig. 3). Finally, the DNase I-digested nuclei were pelleted again, washed and resuspended in 500 μl PBS.

Labelling of nascent DNA

A standard nuclear DNA synthesis reaction (Krude, 1995) contained 30 μl of the suspension of permeabilised HeLa cells and 20 μl of a buffered nucleotide-mix, yielding final concentrations of: 40 mM K-Hepes, pH 7.8, 4 mM $MgCl_2$, 3 mM ATP, 0.1 mM each of GTP, CTP, UTP, 25 μM dATP, 0.1 mM each of dGTP and dCTP, 0.25 μM biotin-16-dUTP (all Boehringer Mannheim), 0.5 mM DTT, 40 mM creatine phosphate, 5 μg phosphocreatine kinase (Boehringer Mannheim). The reactions were gently mixed on ice and started by transferring them to 37°C. After an incubation of 10 minutes, the reactions were stopped by diluting them with 500 μl PBS at room temperature.

Immunolabelling

HeLa cells exponentially growing on glass coverslips were taken from the culture medium, washed twice with PBS at room temperature and directly fixed on the coverslips in 4% paraformaldehyde in PBS for 5 minutes at room temperature. The coverslips were then washed with PBS and subsequently permeabilised and blocked in buffer A (PBS, 0.1% Triton X-100, 0.02% SDS, 2% non-fat dried milk) for 1 hour at room temperature.

Nuclei from the stopped DNA labelling reactions and those taken from the extraction procedures were fixed in 4% paraformaldehyde in suspension for 5 minutes at room temperature. They were subsequently pelleted through a 1 ml cushion of 30% sucrose in PBS onto polylysine-coated coverslips (Mills et al., 1989). The coverslips were washed twice in PBS and blocked in buffer A for 1 hour at room temperature.

Coverslips were incubated with affinity-purified primary rabbit antibodies (at a final dilution of 10 ng/μl in buffer A) at 37°C for 1 hour and subsequently washed three times for 10 minutes in buffer A. The coverslips were then incubated with fluorescein-conjugated donkey anti-rabbit IgG (Amersham, diluted 1:200 in buffer A) as a secondary antibody, streptavidin (Amersham, diluted 1:100 in buffer A) and 5 μg/ml Hoechst 33258 in buffer A at 37°C for 1 hour. They were subsequently washed two times for 5 minutes in buffer A, once in PBS and were mounted for immunofluorescence microscopy in 90% glycerol, containing 1 mg/ml *p*-phenylenediamine (Sigma) in PBS.

Immunofluorescence microscopy

Conventional immunofluorescence microscopy was performed on a Nikon Microphot-SA fluorescence microscope. Photographs were taken at a 200× magnification using Ilford XP4 film (ASA 400). Exposure times were 2-4 seconds.

Confocal immunofluorescence microscopy was performed on a Bio-Rad MRC 600 confocal scanning laser microscope, equipped with a krypton-argon laser (Bio-Rad). Images were taken with four runs of the Kalman algorithm at the slow scanning mode (Bio-Rad COMOS-software). The images derived from both channels (fluorescein and Texas red) were recorded simultanously at identical apertures (4 scale units). The contrast of the fluorescein-derived images was linearly stretched by a factor of 2. Merged images were produced in the alternate pixel mode using the unstretched Texas red-derived image and the stretched fluorescein-derived image. The fluorescein-derived image was assessed with a green colour and the Texas red-derived image with a red colour. Superimposition of red-green signals appears in yellow.

Western blotting

Western blotting of electrophoretically separated proteins (Towbin et al., 1979) was performed using polyvinylidene difluoride membranes (PVDF; Millipore) and a semi-dry blotting apparatus. The membranes were incubated with monospecific antibodies at 1 μg/ml in RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.5% desoxycholate, 0.1% sodium dodecylsulfate, 50 mM Tris-HCl, pH 8.0), and the immunocomplexes were stained (Harlow and Lane, 1988) with alkaline phosphatase-conjugated secondary mouse anti-rabbit IgGs (Sigma).

RESULTS

hCdc21 and hCdc46 are localised in interphase nuclei

In this study, we determined by indirect immunofluorescence microscopy the localisations of the Mcm-proteins hCdc21, hCdc46 and P1Mcm3 in human cells. Polyclonal rabbit antibodies specific for hCdc21 and for hCdc46 (Musahl et al., 1995; Burkhart et al., 1995) were purified by affinity chromatography. Monospecificity was confirmed by immunoblotting whole-cell HeLa cell extracts. Anti-hCdc21 antibody detects a major single band at 97 kDa (Fig. 1), corresponding to hCdc21 (Musahl et al., 1995). The generation of monospecific anti-hCdc46 and anti-P1Mcm3 antibodies has already been reported (Burkhart et al., 1995).

The affinity-purified antibodies were used to determine the intracellular localisation of the human Mcm proteins hCdc 21, hCdc46 and P1Mcm3. HeLa cells were grown on glass coverslips, fixed and analysed by conventional indirect immunofluorescence microscopy (Fig. 2). As a reference, images of the cell morphology (Fig. 2A,D) and of the DNA stained with Hoechst 33258 (Fig. 2B,E) are provided. The hCdc21 protein

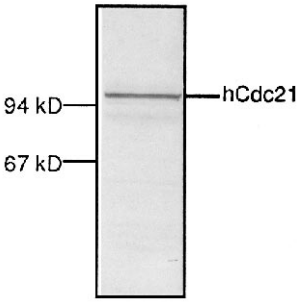

Fig. 1. Characterisation of the monospecific anti-hCdc21 antibodies. Polyclonal anti-hCdc21 antisera (Musahl et al., 1995) were affinity purified as described in Materials and Methods. A whole-cell extract from HeLa cells was separated on an SDS-polyacrylamide gel, blotted and probed with the purified monospecific anti-hCdc21 antibodies. Molecular sizes and the position of hCdc21 protein are indicated. For a characterisation of monospecific anti-hCdc46 and anti-P1Mcm3 antibodies, see Burkhart et al. (1995).

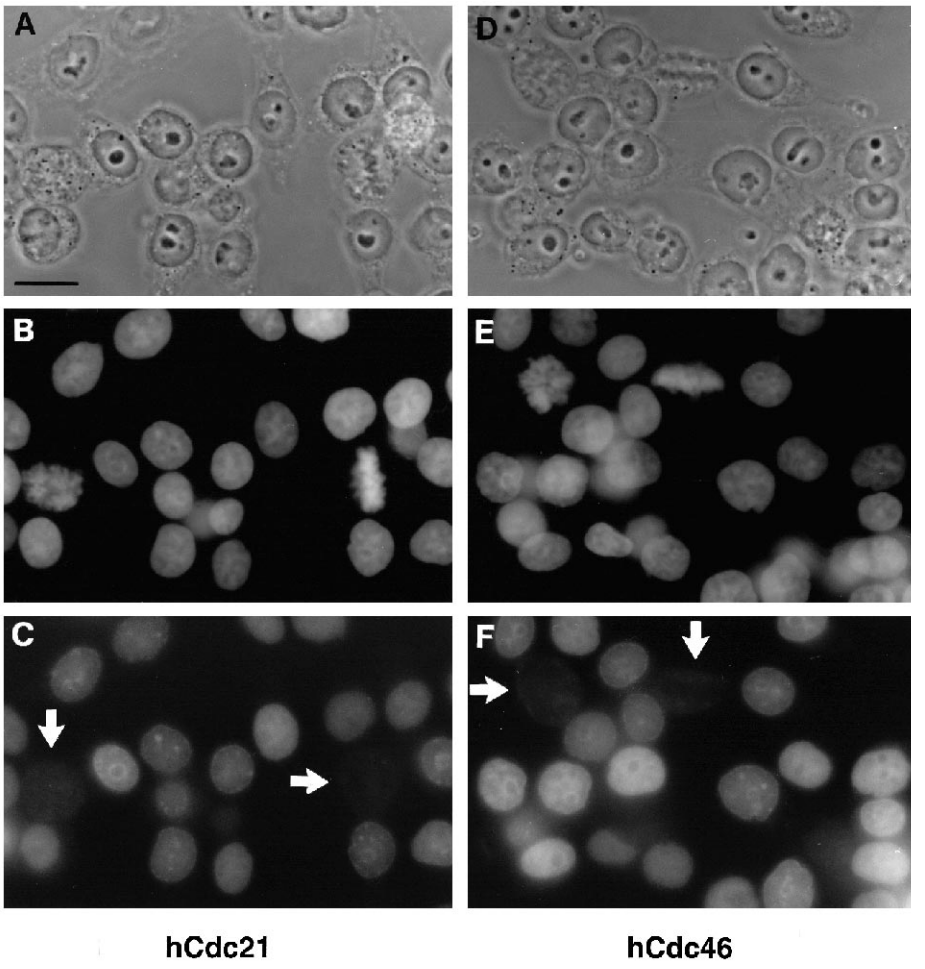

Fig. 2. Human Cdc21 and Cdc46 proteins are localised to the nucleus throughout interphase, and are excluded from mitotic chromatin. HeLa cells were grown on glass coverslips, fixed and viewed by conventional immunofluorescence microscopy. A typical selection of cells is presented: (A,D) phase contrast image; (B,E) DNA stained with Hoechst 33258; (C) visualisation of hCdc21 and (F) of hCdc46 using monospecific polyclonal antibodies and fluorescein-linked secondary IgGs. Arrows in C and F indicate mitotic cells in prophase (left) and metaphase (right). Bar, 10 μm.

is localised to the nucleus in all interphase cells (Fig. 2C). Differences of the staining patterns of individual HeLa cell nuclei within the population of interphase cells are apparent at two particular levels. First, the intensity of hCdc21 immunofluorescence varies between individual HeLa cell nuclei. Second, a discrete strong staining pattern is superimposed on an even nuclear staining in some interphase nuclei (Fig. 2C), occurring at a frequency of approximately 25% in the population of interphase cells (data not shown). The sites of this discrete staining often surround or coincide with the nucleoli and the nuclear periphery. This pattern is reminiscent of the localisation of heterochromatin in the nucleus and resembles further the localisation of sites of DNA replication during late stages of the S-phase (Nakamura et al., 1986; Nakayasu and Berezney, 1989). In mitotic cells, hCdc21 is barely detectable in the cytoplasm and cannot be detected on the condensed mitotic chromatin (Fig. 2C; arrows).

hCdc46 protein is also localised to the nucleus in all interphase cells (Fig. 2F). It has a similar complex immunostaining to that described above for hCdc21. In the cytoplasm of mitotic cells hCdc46 is barely detectable and it is clearly absent from the condensed chromatin in pro- and metaphase (Fig. 2F, arrows) as well as in ana- and telophase (data not shown). A weak and homogeneous nuclear staining of hCdc46 reappeared at the late telophase/G_1-phase boundary (data not shown).

We furthermore analysed the intracellular localisation of P1Mcm3 protein and observed the same intranuclear staining pattern as described here for hCdc46 (data not shown), in agreement with previously published data (Burkhart et al., 1995; Schulte et al., 1995).

A fraction of hCdc21 and hCdc46 is bound to chromatin

In vertebrate cells, Mcm proteins appear to exist in two populations. One population is bound to a nuclear structure in G_1 and early S-phase, while another population appears to be nucleosolic and can be extracted from nuclei by nuclear membrane permeabilisation (Kimura et al., 1994; Burkhart et al., 1995; Musahl et al., 1995; Todorov et al., 1995). The granular localisation of hCdc21, hCdc46 and P1Mcm3 in a subset of interphase nuclei reported above could therefore be due to proteins bound to a particular struture, probably chromatin. We tested this hypothesis and extracted HeLa cell nuclei by various treatments and analysed the residual structures by immunofluorescence microscopy (Fig. 3). A treatment of isolated nuclei with hypotonic buffer removes free nucleosolic proteins from the nuclei, such as DNA polymerases and other replication proteins (Li and Kelly, 1984), but proteins bound to a nuclear structure are retained at their location (Krude, 1995). Hypotonically extracted HeLa nuclei retain a strong immunostaining of hCdc21 (Fig. 3C) and hCdc46 (Fig. 3F) that corresponds to discrete subnuclear sites in some inter-

phase nuclei. Importantly, the background level of evenly stained nuclear protein in many interphase nuclei as seen in Fig. 2 is lost by this treatment. An identical immunostaining of hCdc21 and hCdc46 was observed when the isolated nuclei were extracted with Triton X-100 (data not shown). It has previously been shown by immunoblotting that the eluted nuclear proteins contain considerable amounts of hCdc21, hCdc46 and P1Mcm3 (Burkhart et al., 1995; Musahl et al., 1995). Together, these data show that a fraction of hCdc21 and hCdc46 is bound to a particular nuclear structure at discrete locations, as it was previously demonstrated for the other Mcm proteins BM28/Mcm2 (Todorov et al., 1995) and P1Mcm3 (Kimura et al., 1994; Burkhart et al., 1995).

We next determined whether this population is bound to chromatin or to another nuclear structure. Isolated nuclei were extensively treated with DNase I and the remaining nuclear structures were analysed by immunofluorescence microscopy (Fig. 3G-L). DNase treated nuclei contain considerably less DNA than untreated nuclei (compare Fig. 3B,E with H,K; also see Materials and Methods) and, importantly, contain only trace amounts of hCdc21 and hCdc46 (Fig. 3I,L). Furthermore, the localised granular staining pattern of these proteins in untreated nuclei is lost upon DNase treatment (Fig. 3I,L). Similar results were obtained for P1Mcm3 (data not shown). These data are corroborated by the observations of Burkhart et al. (1995) who showed by immunoblotting that human P1Mcm3 and hCdc46 become solubilised following DNase I digestion of HeLa nuclei. Furthermore, hCdc21 can also be solubilised from isolated chromatin by DNase I digestion (C. Musahl, unpublished observation). We therefore conclude that hCdc21, hCdc46 and P1Mcm3 are bound to chromatin at discrete locations in a subset of interphase cells.

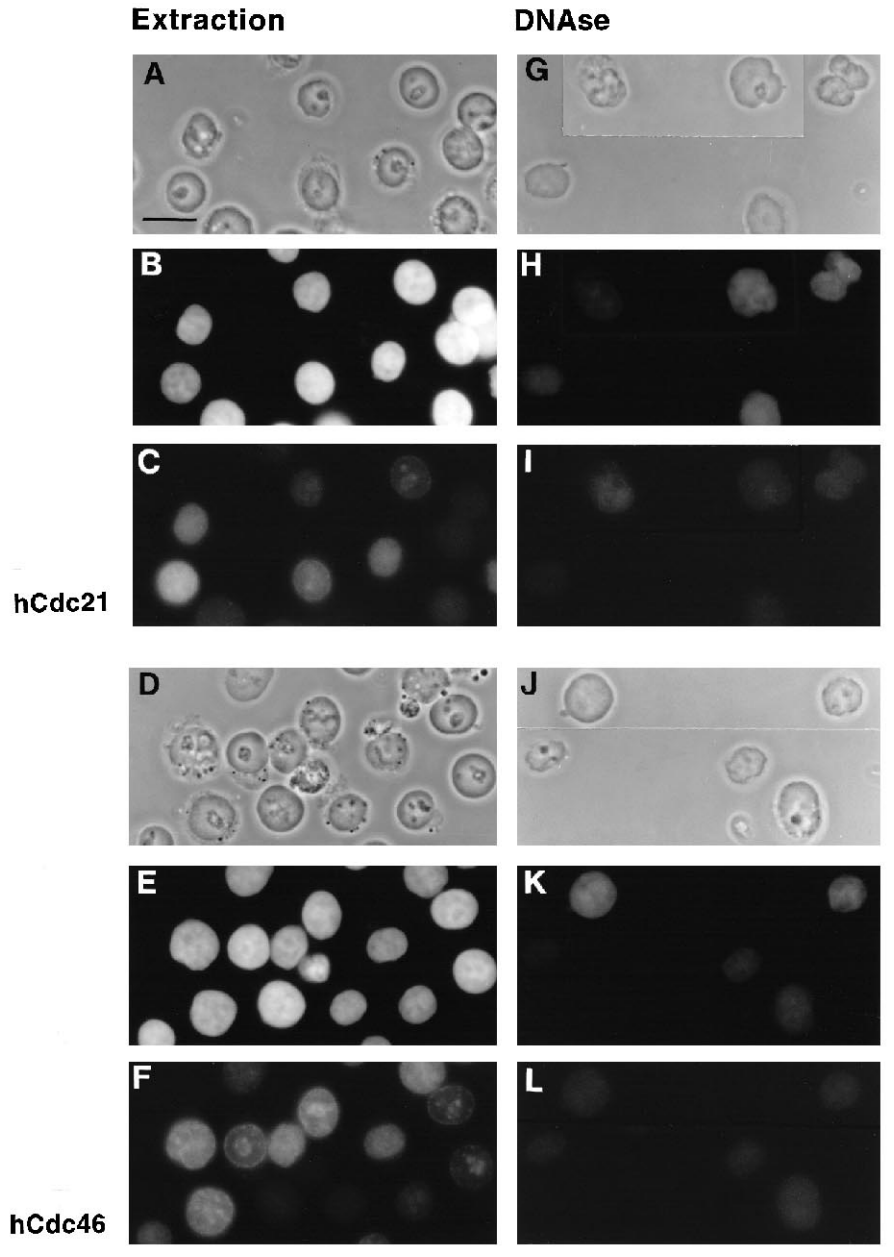

Fig. 3. hCdc21 and hCdc46 proteins are bound to chromatin in a subset of interphase nuclei. Isolated HeLa cell nuclei were treated before fixation either with hypotonic buffer to release free nucleosolic protein (A-F), or with DNase I to solubilise chromatin and associated protein (G-L). Nuclei were subsequently fixed, spun onto coverslips and analysed. Representative selections of nuclei are presented: (A,D,G,J) phase contrast image; (B,E,H,K) DNA stained with Hoechst 33258; (C,I) visualisation of hCdc21 and (F,L) of hCdc46 by indirect immunofluorescence microscopy. Nuclei extracted with hypotonic buffer are shown in (A-F). Similar results were obtained by treating isolated nuclei with 0.1% Triton X-100 (not shown). Nuclei treated with DNase I are shown in G-L. These images were produced under identical conditions to those in A-F. Note the strongly reduced signal intesity for DNA in DNase-treated nuclei (H, K), in comparison to untreated nuclei (B,E). Only background levels of hCdc 21 (I) and hCdc46 (L) were seen in all DNase I-treated nuclei. Bar, 10 μm.

hCdc21 is displaced from chromatin at the sites of DNA replication

We next tested whether or not these discrete locations of the Mcm-proteins correspond to the intranuclear sites of DNA replication. We therefore used high resolution confocal laser scanning microscopy to compare the intranuclear sites of chromatin-bound hCdc21, hCdc46 and hMcm3 proteins with replication foci in hypotonically eluted HeLa cell nuclei. The dynamic pattern of replication foci during the temporal progression of S-phase is shown in Fig. 4A. Individual nuclei that represent subsequent stages of S-phase were aligned according to the criteria of Nakayasu and Berezney (1989). This succession of patterns during S-phase was confirmed by cell cycle synchronisation experiments (data not shown). In the same nuclei, we simultaneously determined the localisation of hCdc21 protein by indirect immunofluorescence (Fig. 4B). To correlate precisely the locations of hCdc21 and of replication foci within the same nucleus, the signals derived from both channels were merged (Fig. 4C).

Some non-replicating nuclei show a bright staining of hCdc21 (Fig. 4, nucleus a) that is slightly weaker at the nucleoli. This staining pattern occurs in about 10% of exponentially growing HeLa cells (data not shown). Using synchronised cells, it occurs only in late G_1 cells, and is absent from a G_2 population (data not shown). We conclude that the uppermost nucleus in Fig. 4A is in late G_1, probably at the G_1/S-boundary.

An almost identical hCdc21-immunostaining is detected in very early S-phase nuclei (b) where a few small DNA replication foci are located in the central euchromatin. At a slightly later stage of early S-phase, many small replication foci are dispersed throughout the euchromatic regions of the nucleus (c). Here, the hCdc21 staining has significantly changed. It appears to be already lost from central euchromatic regions of the nucleus, where DNA replication has initiated. However, Cdc21 is clearly still localised at the heterochromatin and the boundaries between euchromatin and heterochromatin, which will be the sites of DNA replication at later stages of S-phase. When the next stage of S-phase is reached, the hCdc21 staining has again significantly changed (d) and is now predominant only in the heterochromatin at the nuclear periphery and at nucleolar regions. hCdc21 is clearly absent from euchromatin, which at this stage has already been replicated (d).

During mid S-phase, replication foci surround and begin to coincide with the locations of heterochromatin in the nucleus (e). At this stage, the location of the remaining hCdc21 coincides with heterochromatin at the nuclear periphery and in the central parts of the nucleoli. During late S-phase, these heterochromatic regions are replicated (f). At this stage then, only trace amounts of hCdc21 are detectable within the central heterochromatin that is the last DNA replicated in S-phase. At these stages of S-phase it is most apparent that hCdc21 does not co-localise with the replication foci. However, it rather appears that discrete sites of hCdc21 immunostaining are directly adjacent to replication foci and in late S-phase very large foci sometimes seem to directly contact the sites of hCdc21 (note the little yellow signal in nucleus e; however, it cannot be ruled out that this yellow signal results from a partial overlap of actually non-coincident sites within the optical section). At the last stage of S-phase and in the G_2-phase of the cell cycle, no hCdc21 can be detected in HeLa cells (not shown).

In summary, at each stage of S-phase the intranuclear sites of hCdc21 protein appear to coincide with the location of unreplicated chromatin. The respective sites of DNA replication and hCdc21-protein do not co-localise, but are located

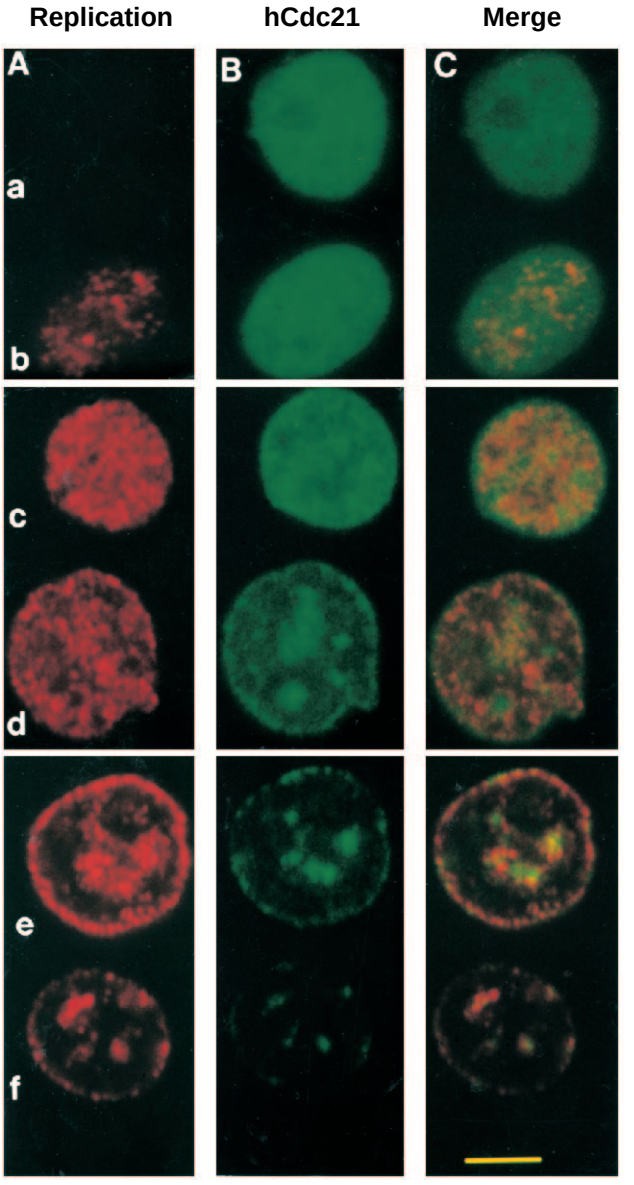

Fig. 4. Bound hCdc21 protein is displaced from replicating chromatin during S-phase. (A) Visualisation of replication foci by confocal immunofluorescence microscopy. Permeabilised HeLa cells were pulse-labelled with biotin-16-dUTP and nascent DNA was visualised by Texas red-linked streptavidin. Nuclei are aligned according to the temporal progression of S-phase (Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Fox et al., 1991; Hassan and Cook, 1993): (a) late G_1, (b) very early S, (c) early S, (d) advanced early S, (e) mid S and (f) late S. (B) Visualisation of hCdc21 in the same nuclei by monospecific antibodies and fluorescein-linked secondary antibodies. (C) Localisation of hCdc21 in respect to replication foci in the same nuclei by merging the images in A and B. A linear contrast stretch (2×) was used for the fluorescein-derived signal in B and C. Bar, 5 μm.

adjacent to each other. hCdc21 is apparently displaced from its location on chromatin at the time of its replication.

P1Mcm3 and hCdc46 are displaced from replicating chromatin

In the next set of experiments, we also compared the intranuclear localisations of P1Mcm3 and hCdc46 proteins (Burkhart et al.,1995) in respect to replication foci during the temporal progression of S-phase.

The localisation of P1Mcm3 relative to replication foci during S-phase progression is shown in Fig. 5. P1Mcm3 appears to be bound to chromatin at the G_1/S-phase boundary (Fig. 5, nucleus a), as has been described above for hCdc21 (cf. Fig. 4). During replication, P1Mcm3 is displaced from chromatin and, like hCdc21, it does not co-localise with replication foci (b-d). P1Mcm3 is absent from replicated chromatin at late S-phase (e) and G_2-phase (data not shown).

The immunostaining pattern of hCdc46 in S-phase nuclei

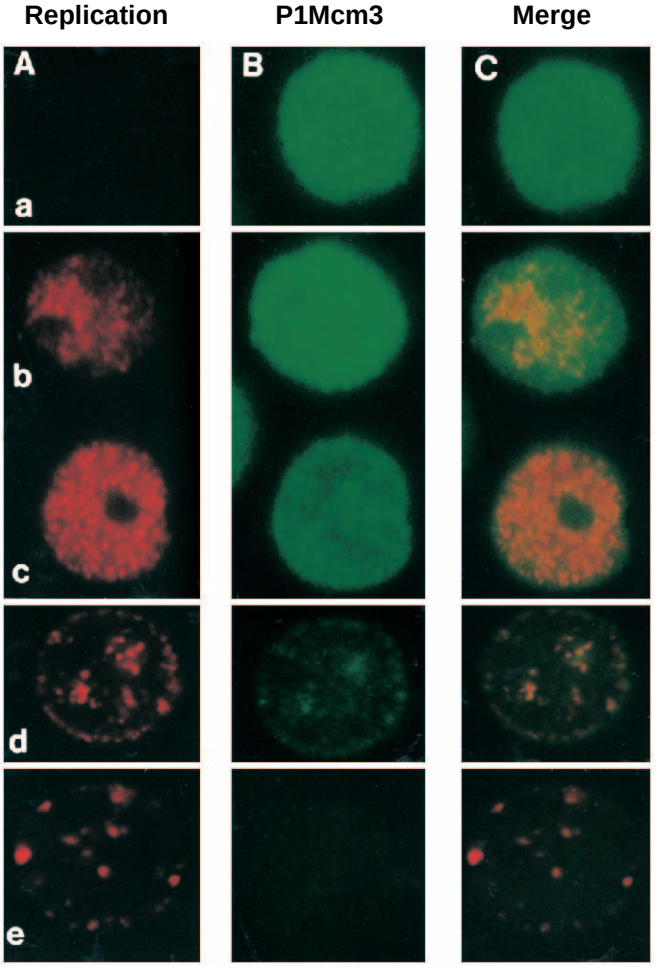

Fig. 5. Bound P1Mcm3 protein is displaced from replicating chromatin during S-phase. (A) Visualisation of replication foci in HeLa cell nuclei. Nuclei are aligned according to the temporal progression of S-phase as in Fig. 4: (a) late G_1, (b) very early S, (c) early S, (d) mid S and (e) very late S. (B) Visualisation of P1Mcm3 in these nuclei by monospecific antibodies and fluorescein-linked secondary antibodies. (C) Merge of the images in A and B. A linear contrast stretch (2×) was used for the fluorescein-derived signal in B and C. The magnification is identical to that in Fig. 2.

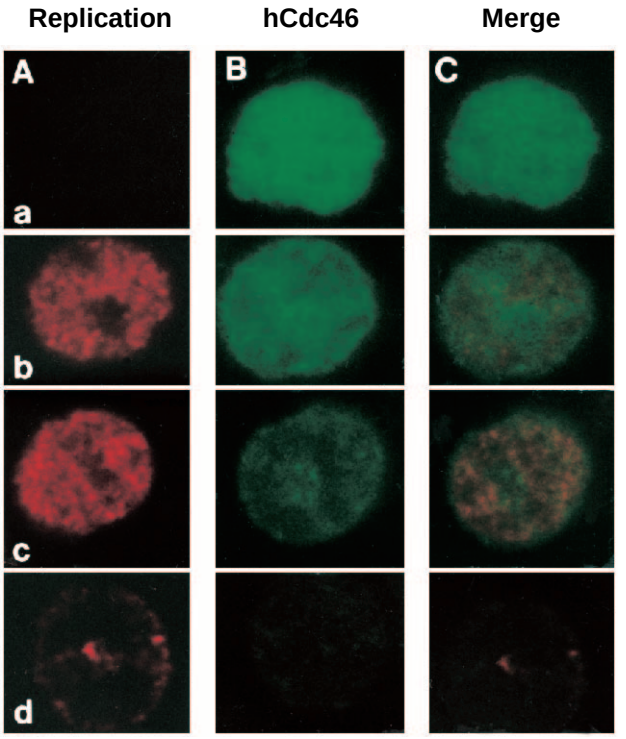

Fig. 6. Bound hCdc46 protein is displaced from replicating chromatin. (A) Visualisation of replication foci in HeLa cell nuclei. Nuclei are aligned according to the temporal progression of S-phase as in Fig. 4: (a) late G_1, (b) early S, (c) advanced early/mid S and (d) late S. (B) Visualisation of hCdc46 in these nuclei by monospecific antibodies and fluorescein-linked secondary antibodies. (C) Merge of the images in A and B. A linear contrast stretch (2×) was used for the fluorescein-derived signal in B and C. The magnification is identical to Fig. 2.

(Fig. 6) is similar to the immunostaining patterns described above for hCdc21 and for P1Mcm3 (cf. Figs 4 and 5). hCdc46 is bound to chromatin at the G_1/S-phase boundary (Fig. 6, nucleus a), does not co-localise with replication foci and it is displaced from chromatin during replication (b-d).

In summary, these high resolution immunofluorescence studies indicate that the human Mcm proteins Cdc21, Cdc46 and P1Mcm3 all appear to have a similar behaviour during the somatic cell cycle. They apparently bind to unreplicated chromatin in a homogenous way before the onset of S-phase, they do not co-localise with replication foci during S-phase and they are displaced from their sites on chromatin when it is replicated. Consequently, replicated chromatin in late stages of S, in G_2 and during mitosis does not contain bound hCdc21, hCdc46 or P1Mcm3.

DISCUSSION

In this paper, we used high resolution confocal microscopy to determine the subnuclear sites of the human Mcm proteins Cdc21, Cdc46 and P1Mcm3 in comparison to the sites of replicating DNA in human cell nuclei. hCdc21, hCdc46 and P1Mcm3 bind to chromatin before the onset of DNA replication, but they do not co-localise with replication foci. Instead, subnuclear sites of chromatin-bound Mcm proteins coincide

with the sites of unreplicated chromatin. During progression of S-phase hCdc21, hCdc46 and P1Mcm3 are displaced from their site on chromatin at the time when it is replicated. Consequently, replicated chromatin does not contain bound hCdc21, hCdc46 or P1Mcm3 during S-phase, G_2 and mitosis.

The involvement of the replication protein Mcm3 in restricting DNA replication to a single cycle has recently been demonstrated in *Xenopus laevis* egg extracts (reviewed by Su et al., 1995). Our immunofluorescence data thus indicate that the human Mcm-proteins Cdc21, Cdc46 and P1Mcm3 behave in the way expected for a 'license to replicate' because they are present only on unreplicated chromatin. They are displaced from replicating chromatin, consistent with a role in allowing only a single round of DNA replication.

Chromatin bound vs nucleosolic Mcm proteins

Previous immunofluorescence studies have shown that the proteins BM28/Mcm2 and P1Mcm3 exist in interphase nuclei in two populations, a nucleosolic and a structure-bound population (Kimura et al., 1994; Todorov et al., 1995; Schulte et al., 1995). In this paper, we demonstrate by immunofluorescence that hCdc21 and hCdc46 proteins also exist as two populations in HeLa cell nuclei. The first one is stably bound to chromatin at discrete sites during S-phase. A second population is nucleosolic and not associated with a particular structure. It is present throughout interphase, but not during mitosis, and is eluted from the nuclei under the experimental conditions used. These microscopical data are entirely consistent with biochemical evidence supporting the existence of two populations each of hCdc21, hCdc46 and P1Mcm3 proteins in human cells (Burkhart et al., 1995; Musahl et al., 1995). A similar conclusion has been reached by other authors concerning the intranuclear localisation of murine Mcm3/P1 (Kimura et al., 1994; Starborg et al., 1995), *Xenopus* Mcm3 (Madine et al., 1995a,b; Chong et al., 1995; Kubota et al., 1995) and human Mcm2/BM28 (Todorov et al., 1995). However, Kimura et al. (1994) further reported that murine Mcm3 is not solubilised by DNase I. This apparent contradiction is most likely explained by different conditions of the DNase I digestion experiments. Therefore it can be concluded that in vertebrate cells members of the Mcm protein family bind to chromatin in (late) G_1 phase, presumably from a nucleosolic pool and are displaced from chromatin into the nucleoplasm during DNA replication.

In the cases examined so far, the chromatin-bound form is underphosphorylated and hyperphosphorylation correlates with the displacement of these proteins from chromatin (Burkhart et al., 1995; Schulte et al., 1995; Musahl et al., 1995; Todorov et al., 1995). It is therefore possible that phosphorylation of these Mcm-family proteins during DNA replication results in or reflects their displacement from chromatin.

Recently, we presented evidence that the soluble Mcm proteins exist in different protein complexes in human cells. On one hand, P1Mcm3 and hCdc46 co-immunoprecipitate as a dimeric complex from HeLa cell extracts (Burkhart et al., 1995; Schulte et al., 1995). On the other hand, hCdc21 co-immunoprecipitates with two other Mcm-proteins, namely p105Mcm and p85Mcm as a distinct trimeric complex (Musahl et al., 1995). These complexes were isolated from the free nucleosolic, as well as from the chromatin-bound form of the proteins (Burkhart et al., 1995; Musahl et al., 1995). However, under the topographical resolution of the confocal immunofluorescence microscope used in this study, proteins of the two particular human Mcm-complexes are found at apparently similar locations throughout S-phase. These results are consistent with the possibility that the two complexes could in fact belong to a single large 'supercomplex' that is disrupted into the observed two 'subcomplexes' under the experimental conditions of immunopecipitation.

Subnuclear sites of chromatin-bound Mcm proteins

Our results indicate that the chromatin bound forms of hCdc21, hCdc46 and P1Mcm3 undergo a characteristic change of intranuclear locations during temporal progression of S-phase. Not only do these sites fail to coincide with sites of ongoing DNA replication, but they appear to be excluded from sites of replicating and replicated DNA, which is most apparent at later stages of S-phase when discrete sites can clearly be separated. This conclusion is supported by the apparent lack of a yellow signal in the merged images in Figs 4-6. Furthermore, the changes of Mcm3 protein bound to *Xenopus* sperm chromatin during DNA replication in *Xenopus* egg extract (Madine et al., 1995b) are consistent with the immunofluorescence data reported above for the human Mcm-proteins in the somatic cell cycle.

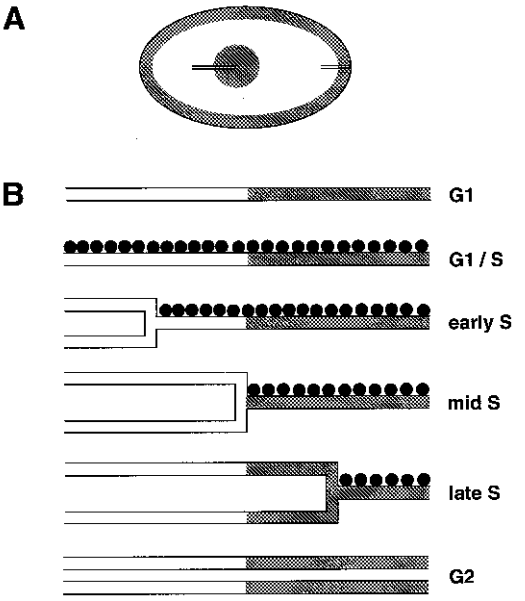

Fig. 7. Summary of the localisations of hCdc21, P1Mcm3 and hCdc46 proteins in respect to the progression of chromatin replication in human cell nuclei. (A) Schematic representation of a HeLa cell nucleus: Early replicating euchromatin is shown in white and late replicating heterochromatin at the nuclear periphery and the nucleoli is shown in grey. The two bars indicate representative sections through chromatin, ranging from eu- (left end) into heterochromatin (right end). (B) Localisation of human Mcm-proteins at these chromatin sections during their replication in S-phase. The Mcm-proteins hCdc21, P1Mcm3 and hCdc46 are represented by filled circles. Early replicating euchromatin is indicated in white and late replicating heterochromatin in grey. Note that this cartoon does not illustrate the movement of a single replication fork, but symbolises the gradual progression of replicated sections of chromatin during S-phase. Unreplicated chromatin is indicated by a single bar and replicated chromatin by a duplicated bar. The progression of replication during S-phase is viewed from top to bottom.

From these data a model can be deduced (Fig. 7) that integrates the locations of the hCdc21, hCdc46 and P1Mcm3 proteins in respect to the sites of DNA replication throughout S-phase. First, these proteins associate with chromatin prior to the onset of DNA replication. Second, during all stages of S-phase the chromatin-associated Mcm-proteins are lost from the intranuclear location that has been replicated at the preceding stage of the S-phase. It can therefore be concluded that these proteins are displaced from chromatin at the sites of DNA replication. At present, displacement of the Mcm-proteins during initiation or during elongation are both consistent with our immunofluorescence data. These data extend recently published observations that a decrease of nuclear Mcm3 and BM28/Mcm2 immunofluorescence is correlated with the extent of replicated DNA in the nuclei of murine, *Xenopus* and human cells, respectively (Kimura et. al., 1994; Kubota et al., 1995; Madine et al., 1995a,b; Chong et al., 1995; Todorov et al., 1995). Our data provide experimental evidence that chromatin associated human Mcm proteins hCdc21, hCdc46 and P1Mcm3 are displaced from regions of replicated chromatin. Therefore, Mcm proteins could provide a means to distinguish between replicated and unreplicated chromatin in vetebrate cell nuclei.

A 'license to replicate'

In *Xenopus*, Mcm3 protein has been implicated in replication licensing (Kubota et al., 1995; Madine et al., 1995a,b; Chong et al., 1995; reviewed by Su et al., 1995). The original model of replication licensing (Laskey et al., 1981; Blow and Laskey, 1986) proposed a positive replication factor that was distributed on DNA only before the start of S-phase and then displaced by replication. However, in a more recent version of the licensing model, Blow and Laskey (1988) proposed that the factor was excluded by the nuclear membrane in order to account for observations that permeabilising the nuclear membrane allows replicated nuclei to replicate again. In contrast, Madine et al. (1995a) have shown that XMcm3 can cross the nuclear membrane before initiation of DNA replication and have further shown that permeabilising the nuclear membrane is required to allow Mcm-proteins to bind to chromatin rather than to enter the nucleus (Madine et al., 1995b). The behaviour of hCdc21, hCdc46 and P1Mcm3 in human cells reported here conforms to the first of these licensing models in terms of a 'license to replicate', as does the behaviour of mouse and *Xenopus* Mcm3 (Kimura et al., 1994; Kubota et al., 1995; Madine et al., 1995a,b; Chong et al., 1995).

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