BM28, a Human Member of the MCM2-3-5 Family, Is Displaced from Chromatin during DNA Replication

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Abstract. We have recently cloned and characterized a human member (BM28) of the MCM2-3-5 family of putative replication factors (Todorov, I. T., R. Pepperkok, R. N. Philipova, S. Kearsey, W. Ansorge, and D. Werner. 1994. J. Cell Sci. 107:253–265). While this protein is located in the nucleus throughout interphase, we report here a dramatic alteration in its nuclear binding during the cell cycle. BM28 is retained in the nucleus after Triton X-100 extraction in G1 and early S phase cells, but is progressively lost as S phase proceeds, and little BM28 is retained in detergent-extracted G2 nuclei. BM28 that is resistant to extraction in G1 nuclei is removed by DNase I digestion, suggesting that the protein is chromatin associated.

In addition, we present evidence for variations in the electrophoretic mobility of BM28 that may reflect post-translational modifications of BM28 during the cell cycle. During mitosis, BM28 is present as a fast-migrating form, but on entry into G1, the protein is converted into a slow-migrating form. With the onset of S phase, the slow-migrating form is progressively converted into the fast form. BM28 is phosphorylated at all stages of the cell cycle, but during interphase the fast form is hyperphosphorylated compared with the slow form. These apparent changes in modification may reflect or effect changes in the nuclear binding of BM28.

The behavior of BM28 is not dissimilar to related proteins in Saccharomyces cerevisiae, such as Mcm2p, which are excluded from the nucleus after DNA replication. We speculate that BM28 may be involved in the control that limits eukaryotic DNA replication to one round per cell cycle.

STUDIES of chromosomal replication have shown that, in many systems, an initiation event at a replication origin is followed by a latent period, during which that origin is inactive. This is demonstrated clearly in eukaryotic cells, where initiation events, which occur throughout S phase, only take place on unreplicated DNA. Replicated chromosomes usually have to pass through mitosis before further initiation events can occur, and thus one round of replication per cell cycle is ensured (for review see Coverley and Laskey, 1994; Diffley, 1994; Stillman, 1994). The nature of the control is not clear but appears to be cis-acting at the level of the template, as cell fusion experiments have indicated that a G1, but not a G2, nucleus can replicate when fused to an S phase cell (Rao and Johnson, 1970). Studies on the effects of nuclear permeabilization on DNA replication in Xenopus egg extracts have suggested that G1 but not G2 nuclei contain “licensing factor,” which acts as a positive signal, permitting DNA replication (Blow and Laskey, 1988; for review see Coverley and Laskey, 1994). In this model, a hypothetical licensing factor binds to chromatin during mitosis and permits DNA replication during the following S phase. The licensing factor is inactivated by the passage of the replication fork, excluded from the nucleus, and its access to the chromatin is blocked by the nuclear membrane until the following mitosis, thus preventing over-replication of DNA during a single cell cycle.

Evidence for a licensing factor–based model would clearly benefit from the identification of a suitable candidate molecule. Recently, a family of proteins has been identified in Saccharomyces cerevisiae and other eukaryotes that has some of the properties expected of licensing factor (for review see Tye, 1994). There are at least five distinct proteins in this family in S. cerevisiae, of which Mcm1p, Mcm2p, Mcm3p (Yan et al., 1991, 1993), and Cdc46p/Mcm5p (Hennessy et al., 1990, 1991; Chen et al., 1992) have been studied in most detail. These proteins share extensive sequence similarity in a central region, but show relatively little similarity in their NH2- and COOH-termi-
nal regions. All the yeast genes so far analyzed are essential, indicating that the proteins cannot substitute for each other’s activity. Conditional mutants show a cell cycle arrest in interphase, S phase execution is affected, and the ability of replication origins to function is reduced, suggesting that the initiation step of DNA replication is altered. Strains bearing the cdc46-1 conditional allele arrest with a single genomic equivalent of DNA, suggesting that there is a global requirement for Cdc46p in chromosome replication (Hennessy et al., 1990, 1991). The biochemical function of these proteins is obscure, but they all contain a “deviant” nucleoside triphosphate (NTP)-binding motif (Koonin, 1993), suggesting that they may be capable of hydrolyzing NTPs. Studies of the cellular localization of these proteins in budding yeast have revealed an interesting cell cycle alteration, which has led to comparisons with hypothetical licensing factor. Mcm2p, Mcm3p (Yan et al., 1993) and Mcm5p/Cdc46p (Hennessy et al., 1990) are found in the nucleus during the G1 phase, but disappear during S phase, and they remain excluded until a late mitotic stage, inviting speculation that disappearance from the nucleus ensures that there is no residual activity to promote further DNA replication.

In addition to S. cerevisiae, relatives of this protein family have been studied in other eukaryotes. In fission yeast, three genes in this family, namely, cdc21+ (Nasmyth and Nurse, 1981; Coxon et al., 1992), nda1+, and nda4+ (Miyake et al., 1993), have so far been identified from screens for conditional mutations that have a cell cycle-arrest phenotype, and each gene may be homologous to a budding yeast relative. Strains mutant in these genes are also affected in S phase execution, similar to the situation in S. cerevisiae. Higher eukaryotic members of this gene family have also been discovered, starting with the murine P1 protein, closely related to Mm3p of budding yeast (Thommes et al., 1992; Hu et al., 1993; Bucci et al., 1993). While this manuscript was being prepared, Kimura et al. (1994) reported an analysis of the cell cycle changes in the nuclear binding and phosphorylation of the P1 protein.

We have recently identified a human member of the family, called BM28, which is most closely related to S. cerevisiae Mm3p and the fission yeast nda1+ gene product (Todorov et al., 1994). Microinjection of an antibody raised against recombinant BM28 into synchronized mouse NIH3T3 or human IMR-90 and HeLa cells presents evidence for the involvement of the protein in DNA replication. When injected during G1 phase, the anti-BM28 antibody inhibits the onset of subsequent DNA synthesis as tested by the incorporation of bromodeoxyuridine (BrdU). However, in contrast to the results obtained for Mm3p, our initial immunocytochemical data for BM28 had shown a nuclear localization of the protein throughout interphase.

In this report, we show that although always localized in the nucleus, prefixation treatment with nonionic detergents reveals that BM28 has different extractability during the cell cycle, being tightly bound to a nuclear component only during the G1 and early S phases of the cell cycle. In addition, this binding seems to be related to chromatin integrity, as DNase I digestion releases the majority of bound BM28 from G1 and early S phase nuclei. We also document posttranslational modification changes in the BM28 protein, which may be relevant to this behavior. Our results suggest that BM28 is chromatin bound during G1, but is displaced during DNA replication and cannot rebind until an event in late mitosis. As such, BM28 could be involved in the control that allows the replication apparatus to distinguish replicated from unreplicated chromatin.

Materials and Methods

Cell Culture

HeLa, mouse NIH3T3 cells (Todorov et al., 1994), and buffalo rat BRL3A cells (Dulak and Temina, 1973) were grown in DME supplemented with 10% FCS and 1% antibiotic/antimycotic solution (GIBCO BRL, Paisley, UK). HeLa cells were synchronized in mitosis using mitotic shake-off, following or not following nocodazole treatment as described earlier (Todorov et al., 1994; see also Johnson et al., 1993). For a mitotic arrest, 40 ng/ml of nocodazole was added for 10-12 h. These cells were used also as a G1 source 4-6 h after replating. For another G1 source, cells were arrested in early G1 by 20 mM lovastatin (generous gift of Dr. A. Alberts, Merck Research Laboratories, Rahway, NJ) for 24 h, followed by a release in 1 mM mevalonlic acid (Sigma Chemical Co, St. Louis, MO) for at least 2 h (Koomarsi et al., 1991; Jakobiaski et al., 1991). In our hands, the cells remained in G1 phase up to 7-8 h after the mevalonlic acid release and then entered synchronously into S phase, giving >90% of BrdU-positive cells (see below) 10 h after the release. This time point was used as a source of early S cells in some experiments. Cells were also arrested in early S phase with 2 mM hydroxyurea for 16 h and released by several washes in fresh medium (Todorov et al., 1994). After this block, the cells remained in S phase up to 7-8 h after the release as judged by BrdU incorporation. Cells were considered to be in early S phase 2 h after the release from hydroxyurea and in late S phase 6 h after the release. For most of the experiments, a cell cycle point more than one method of synchronization was used, to avoid artifacts due to the action of the drugs. In all experiments, the efficiency of cell synchronization was assessed by BrdU labeling followed by immunostaining with anti-BrdU antibody. The cells were pulsed for 15 min with 20 μM BrdU, and 1 μM fluorouridine (Bravo and McDonald-Braun, 1987) before extraction and fixation.

Cell Extractions

The extractions were performed directly on cells grown on coverslips (in situ extractions) or on cells in suspension. The cells were washed with PBS and with cytoskeleton (CSK) buffer (10 mM Pipes, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2) (Fey et al., 1986). Then the cells were extracted with CSK buffer containing 0.5% Triton X-100, 0.5 mM PMSF, 10 μg/ml of each of leupeptin and aprotinin (Boehringer Mannheim, GmbH, Mannheim, Germany) for 5 min at 20°C. In the case of nuclelease digests, 100 μg/ml DNase I and/or RNase A (Sigma Chemical Co.) were added to the extraction buffer. After a wash in CSK buffer, the coverslips were fixed in 4% paraformaldehyde in CSK buffer for 30 min on ice and processed for immunofluorescence.

For biochemical analysis the cells were scraped in PBS containing 2 mM MgCl2 and 0.5 mM PMSF, washed with PBS and CSK buffer, and extracted as described above. The cells were digested for 5 min at 20°C in 5 μl CSK buffer containing Triton X-100, protease inhibitors, and/or nucleases. The remnants and extracts were separated by centrifugation at 700 g for 5 min. The pellets were washed once with CSK buffer, and the supernatants were added to the corresponding extract fractions. All samples were finally lyed in electrophoresis sample buffer for further analysis.

Immunofluorescence Microscopy

For immunolocalization, all the steps, except the final washes, were performed in a blocking solution (1% BSA, 100 mM lysine in PBS). Paraformaldehyde-fixed cells were first blocked for 30 min and then incubated for 2 h at 20°C with 20 μg/ml affinity-purified anti-BM28 antibody (Todorov et al., 1994). In most of the experiments, both antibodies against the NH2 and COOH-terminal regions of BM28 were used and displayed the same staining pattern. A FITC- or Texas red-conjugated goat anti-
Phosphorylation Analysis

To study the phosphorylation of the BM28 protein, HeLa cells were labeled with $^{32}$Porthophosphate (ICN Biomedicals, Inc., Costa Mesa, CA) in 35-mm petri dishes as follows. Cells synchronized in different phases of the cell cycle as described above were washed with phosphate-free medium based on DME (GIBCO) and preincubated for 30 min at 37°C with phosphate-free medium containing 5% dialyzed FCS. $^{32}$POrthophosphate was added to reach 1 mCi/ml, and cells were incubated for 2 h at 37°C. The cells were rinsed with cold PBS in the petri dishes and solubilized in lysis buffer containing 20 mM Na phosphate as described above. The lysate was immunoprecipitated with an anti-BM28 antibody. The immunoprecipitate was eluted, resolved on SDS gels, and immunoblotted. The $^{32}$P labeling was detected by autoradiography.

Results

Cell Cycle-dependent Changes in the Nuclear Retention of BM28

Our previous immunolocalization and immunoblotting data suggested that neither the level nor localization of the human BM28 protein varies dramatically during the cell cycle. In cell fractionation experiments, however, we noticed that a proportion of BM28 is readily lost from permeabilized cells, suggesting that the protein may not be tightly bound to nuclear components (see below). Given that the related Mcm2p in S. cerevisiae is located in the nucleus only during the interval from mitosis to early S phase, we investigated whether there might not be more subtle changes in BM28 distribution during the cell cycle. Instead of fixing cells in the normal way for indirect immunofluorescence, we first permeabilized the cells with a nonionic detergent directly on coverslips just before fixation (termed here “in situ extraction”). This procedure is based on the methods used to extract soluble cellular proteins as an initial step of in situ preparations of nuclear matrix or other nuclear structures (Staufnbiel and Depper, 1994; Fey et al., 1986; Nakayasu and Berezney, 1989; Gerdels et al., 1994). With this method, the result was strikingly different: cells showed considerable variation in nuclear staining with the anti-BM28 antibody, implying that the antigen could be efficiently extracted from a subset of cells (Fig. 1). Some cells showed an intensity of nuclear fluorescence similar to that found with normally fixed cells (Fig. 1C, arrowheads), whereas others showed hardly any staining with the anti-BM28 antibody (Fig. 1C, small arrows). In the initial experiments, we used three cell lines of different origin: buffalo rat BRL3A (Fig. 1C), human HeLa cells (Figs. 2, 3, 8, and 10), and mouse NIH3T3 (not shown); all gave similar results. For extraction, we usually used 0.5% Triton X-100, but identical results were obtained with other nonionic detergents, such as NP-40.

Given the yeast results with Mcm2p, an obvious explanation for this variation is that BM28 is more sensitive to detergent extraction in certain phases of the cell cycle. To test this prediction, unsynchronized HeLa cells were double stained for BM28 and DNA, and the images (Fig. 2, A and B) were collected using a charged-coupled device camera to allow quantitation of the fluorescence images (see Materials and Methods). Analysis of several fields showed an inverse correlation between the average FITC fluorescence intensity (proportional to BM28 concentration) and the integrated DAPI fluorescence intensity (proportional to DNA content) of individual nuclei (Fig. 2C).
This suggested that BM28 is more readily extracted from cells that have replicated their DNA. In a second experiment, we synchronized HeLa cells in G1 with lovastatin or in early S phase with hydroxyurea and examined the extractability of BM28 at different time points after release from the inhibitors. In cells synchronized in G1 (2–6 h after release from a lovastatin block; Fig. 3 B) or early S phase cells (not later than 2 h after release from a hydroxyurea block, Fig. 3 C; or 10 h after release from a lovastatin block, not shown) the BM28 antigen is nuclear, showing an intensity and pattern of staining comparable to that of nonextracted cells. In such cells, the pattern of BM28 staining is almost indistinguishable from the distribution of DNA revealed by DAPI (Fig. 2, A and B; see Fig. 8, A and B). Cells released for >4 h from the hydroxyurea block show a less uniform distribution of BM28 (Fig. 3 D), and 6 h after the release, relatively little BM28 can be detected in the nucleus (Fig. 3 E). At this stage, the remaining antigen is concentrated in “speckles” and in the peri-nucleolar region. Cells released from the hydroxyurea block for 8–10 h, which are in G2, often show faint BM28 staining in the nuclear envelope region (Fig. 3 F). Thus, as S phase proceeds, there is a considerable reduction in the nuclear retention of BM28, although a small proportion of BM28 may be refractory to extraction even in the G2 phase. This may represent a nuclear compartment to which a minor amount of BM28 is bound throughout interphase. Overall, these results are reminiscent of those obtained with Mcm2p in S. cerevisiae, in that during S phase there appears to be a reduction in the affinity of BM28 for some nuclear structure. Unlike the situation in yeast cells, however, the human protein remains in the nucleus during G2.

Analysis of BM28 in Cell Extracts

To determine whether the changes detected by indirect
The intensity of BM28 staining in extracted cells is inversely proportional to a parallel staining for DNA. HeLa cells were extracted with Triton X-100, fixed, and stained as shown in Fig. 1. The images for BM28 (A) and DNA (B) staining were taken with a CCD camera and analyzed as described in Materials and Methods. In C, the data points on the graph represent individual nuclei and relate the mean FITC fluorescence intensity (proportional to BM28 concentration) to the integrated DAPI intensity (proportional to DNA content) for a specific nucleus shown in A and B. The axes are calibrated in arbitrary units, and a simple linear regression line is shown fitted by the method of least squares ($r = -0.9$). The analysis shows that nuclei with a high DNA content (presumably G2 nuclei) have a lower BM28 concentration than nuclei with a lower DNA content (G1 nuclei). Analysis of six other randomly selected fields gave a similar result (not shown). Bar in B, 10 μm.

**BM28 is a Phosphoprotein**

We investigated the possible role of phosphorylation as a posttranslational modification of the BM28 protein. HeLa cells were synchronized in various stages of the cell cycle and grown in the presence of $[^32P]$orthophosphate. BM28 was isolated by immunoprecipitation and analyzed by SDS-PAGE and Western blotting (Fig. 5). This analysis clearly shows that BM28 is a phosphoprotein, and both fast and slow forms of the protein are phosphorylated. During the whole length of interphase, the fast form of the protein appears to be approximately fourfold more phosphorylated than the slow form.
Figure 3. The extractability of BM28 increases through the cell cycle. HeLa cells were synchronized in G1 2 h after a release from a lovastatin block (B), and in S phase with hydroxyurea. The cells were considered to be in early S phase 2 h after release from a hydroxyurea block (C), in mid-S phase 4 h after release (D), and in late S phase 6 h after release (E). The cells were considered to be in G2 phase 10 h after release from a hydroxyurea block (F). All stages were assessed by parallel BrdU staining (see Materials and Methods). The cells were fixed and stained for BM28 as in Fig. 1. The protein is tightly bound in the nuclei during G1 (B) and early S (C) phases, more extractable during mid-S (D) and easily extractable in late S (E) and G2 (F) phases. Neither of the inhibitors affects the pattern of BM28 staining compared with nonsynchronized cells (A). Bar, 10 μm.
Different isoforms of BM28 during the cell cycle. HeLa cells were synchronized in mitosis with nocodazole (lane 1), in G1 2 h after release from a lovastatin block (lane 2), in early S phase 2 h after release from a hydroxyurea block (lane 3), and in G2 10 h after release from hydroxyurea (lane 4). The cells were lysed in SDS electrophoresis sample buffer, separated in a 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose. BM28 was identified using a reaction with its specific antibody and an alkaline phosphatase-conjugated second antibody. The migration of Biorad protein standards is indicated. Approximate proportions of BM28 protein in the two electrophoretic forms are as follows (fast:slow): G1 (<20%; >80%); early S (30%;70%); G2 (40%;60%); M (>80%;<20%).

We also investigated whether the mobility changes shown by BM28 could be directly ascribed to changes in phosphorylation. Digestion of immunoprecipitates of BM28 with λ phosphatase decreased the relative mobility of the fast form to a position close to the slow form (Fig. 6). Such a shift was detected in all phases of the cell cycle. Thus, the dephosphorylated form of BM28 has a lower mobility on SDS-polyacrylamide gels than the hyperphosphorylated form. Although this is opposite to the relationship usually found for phosphorylated proteins, it might be related to the abnormal mobility of BM28 in SDS-polyacrylamide gels (Todorov et al., 1994).

Cell Fractionation of BM28

To corroborate the results obtained by the in situ extraction method, we examined the distribution of BM28 on Western blots after crude cell fractionation. HeLa cells

Figure 4. Different isoforms of BM28 during the cell cycle. HeLa cells were synchronized in mitosis with nocodazole (lane 1), in G1 2 h after release from a lovastatin block (lane 2), in early S phase 2 h after release from a hydroxyurea block (lane 3), and in G2 10 h after release from hydroxyurea (lane 4). The cells were lysed in SDS electrophoresis sample buffer, separated in a 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose. BM28 was identified using a reaction with its specific antibody and an alkaline phosphatase-conjugated second antibody. The migration of Biorad protein standards is indicated. Approximate proportions of BM28 protein in the two electrophoretic forms are as follows (fast:slow): G1 (<20%; >80%); early S (30%;70%); G2 (40%;60%); M (>80%;<20%).

Figure 6. λ-Phosphatase digestion alters the electrophoretic mobility of BM28, shifting the fast form to a slower mobility. Extracts from unsynchronized HeLa cells (lanes 7 and 8) or from cells synchronized in mitosis (lanes 1 and 2), G1 (lane 3 and 4), and S (lanes 5 and 6) phases as in Fig. 4 were immunoprecipitated with an anti-BM28 antibody as described in Materials and Methods. Half of each immunoprecipitate was digested with λ-phosphatase (lanes 2, 4, 6, and 8), while the rest was kept as a control (lanes 1, 3, 5, and 7). The immunoprecipitates were eluted and resolved as in Fig. 4.

Cell Fractionation of BM28

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Figure 5. BM28 is a phosphoprotein. HeLa cells were synchronized in mitosis, G1, and S phases with nocodazole, lovastatin, or hydroxyurea blocks and labeled with [32p]orthophosphate for 2 h as described in Materials and Methods. Mitotic cells (lane 1) were labeled adding the isotope to nocodazole-arrested cells. G1 (lane 2) and early S (lane 3) phase cells were labeled after release from nocodazole or hydroxyurea blocks. For labeling late S/G2 phase cells (lane 4), the [32p]orthophosphate was added 6 h after release from the hydroxyurea block. All cells were lysed and immunoprecipitated with the anti-BM28 antibody. The immunoprecipitate was resolved as in Fig. 4 and blotted onto nitrocellulose. BM28 was identified by an immune reaction (B), and the membranes were exposed for autoradiography (A).

Figure 7. Differential extractability of BM28 during the cell cycle. HeLa cells were synchronized in G1 (lanes 1–3), early S (lanes 4–6), G2 (lanes 7–9), and mitosis (lanes 10–12) as in Fig. 4. An aliquot of the cells was lysed directly in electrophoresis buffer to give total (T) cellular proteins (lanes 1, 4, 7, and 10). The rest was extracted with 0.5% Triton X-100, and the pellet (P; lanes 2, 5, 8, and 11) and the extract (E; lanes 3, 6, 9, and 12) were solubilized in a sample buffer and loaded by a manner to represent equal amounts of cells. The samples were separated and immunoblotted as in Fig. 4. The proportion of BM28 that can be extracted at different cell cycle stages is as follows: G1, 25%; S, 35%; G2, 75%; M, 95%.
Figure 8. The non-detergent extractable form of BM28 is sensitive to DNase I digestion. HeLa cells were synchronized in G1 2 h after release from a lovastatin block. The cells were extracted with 0.5% Triton X-100 alone (A and B) and in the presence of RNase A (C and D) or DNase I (E and F). The cells were fixed and stained for BM28 (A, C, and E) and DNA (B, D, and F) as in Fig. 1. Practically the same pattern of staining was detected also after digestion of early S phase cells (10 h after release from a lovastatin block or 2 h after release from a hydroxyurea block). Bar, 10 μm.

were synchronized by mitotic shake-off, lovastatin, or hydroxyurea, and cells were extracted with Triton X-100 under conditions similar to those used in the in situ extraction experiments. Extracted cells were then separated into pellet (P) and supernatant (E) fractions by centrifugation (Fig. 7). Microscopic examination of the pellet fractions showed that they retained good nuclear morphology. Comparing the immunoblots of the pellet and supernatant fractions, we found that most BM28 remained in the pellet when G1 cells are extracted, while in G2 the majority of the protein is soluble (Fig. 7 legend). In mitotic cells, hardly any BM28 is detectable in the pellet fraction. Thus, in agreement with the in situ extraction results, we find that most BM28 cannot be solubilized by detergent in G1, but a change occurs during S phase to allow the protein to be more readily extracted from detergent-permeabilized cells. In this analysis, we also detected both fast and slow forms of BM28 during interphase. When both forms are present (for instance, in S and G2 cells), the slow form is clearly enriched in the pellet, suggesting that this form is more tightly bound than the fast form (Fig. 7, compare lanes 4 and 5, 7 and 8).
Figure 9. DNase I digestion releases BM28 from G1 and early S phase cells. HeLa cells were synchronized in G1 (A) by lovastatin and in early S phase (B) by hydroxyurea as above. An aliquot of the cells was lysed to give total cellular proteins (lane 1). The rest was separated into equal parts and extracted as in Fig. 8 with Triton X-100 alone (lanes 2 and 3) and in the presence of DNase I (lanes 4 and 5), RNase A (lanes 6 and 7), and DNase I + RNase A (lanes 8 and 9). The loading, separation, and analysis of the pellets (lanes 2, 4, 6, and 8) and extracts (lanes 3, 5, 7, and 9) were essentially as in Fig. 7. The approximate proportion of BM28 that can be extracted after the different treatments is as follows: G1, control: 25%; G1, DNase: 80%; G1, RNase: 20%; G1, DNase and RNase: 80%; S, control: 40%; S, DNase: 80%; S, RNase: 50%; S, DNase and RNase: 75%.

Chromatin Association of BM28

As mentioned above, the general pattern of BM28 immunofluorescence in G1 and early S phase cells is very similar to the staining for DNA with DAPI. This similarity is enhanced in Triton X-100–extracted cells (Fig. 2, A and B; Fig. 8, A and B), suggesting that bound BM28 may be chromatin associated. To examine the nature of the subnuclear structure associated with BM28, we carried out in situ extractions in the presence of RNase A or DNase I. RNase A had almost no effect on the distribution of BM28 (Fig. 8 C), suggesting that RNA-containing structures in the nucleus are not important for retention. In contrast, DNase I treatment resulted in the loss of BM28 staining, leaving only faint “speckled” structures surrounding the nucleoli (Fig. 8 E). In immunoblotting experiments we found similar results (Fig. 9). The association of BM28 with the pellet of detergent-extracted G1 and early S cells was not affected by RNase A digestion, but there was a clear solubilization of BM28 on DNase I treatment. On detergent treatment alone, ~25% of BM28 was released from G1 cells, which is similar to the percentage of total cell proteins released under these conditions. With DNase I, >80% of BM28 was released, while the percentage of total proteins extracted was similar to that obtained with detergent treatment alone, and the core histones were not released (data not shown; Fey et al., 1986). These experiments suggest that the bound form of BM28 is largely associated with chromatin. Similar results have been reported for Mcm2p, where the binding of this protein to G1 nuclei was also shown to be abolished by DNase I digestion (Yan et al., 1993).

We also investigated the nature of the nuclear domain that is labeled by anti-BM28 antibodies after detergent extraction of mid- to late S phase cells (Fig. 3). PCNA, the auxiliary subunit of DNA polymerase δ, shows a characteristic nonuniform distribution in S phase nuclei that reflects discrete sites of DNA replication (Bravo and MacDonald-Bravo, 1987). However, simultaneous staining with BM28 and PCNA indicates that the speckled distribution of BM28 in extracted mid–S phase nuclei does not coincide with the pattern of PCNA staining (Fig. 10, A and B). Similarly, simultaneous labeling with BrdU, to label replication foci, and staining for BM28 reveals patterns that are dissimilar (Fig. 10, C and D). The patterns of staining obtained are widespread, and some overlap in the distribution of PCNA or newly incorporated BrdU with BM28 probably occurs in mid–S phase cells, but the gross distributions seem quite different. We also compared the distribution of BM28 with replication protein A (RPA), which is a single-stranded DNA-binding protein required for DNA replication, repair, and possibly recombination (Kenny et al., 1990; Longhese et al., 1994). In extracted cells, mAbs to the 34- (Fig. 10 F) and 70-kD (results not shown) subunits of RPA show widespread granular staining of the nucleoplasm, similar to results obtained using nonextracted cells (data not shown; Kenny et al., 1990; Adachi and Laemmli, 1992; Cardoso et al., 1993). In extracted G1 nuclei, the pattern of BM28 staining clearly overlaps that of RPA, since both antibodies effectively “paint” the nucleus. Cells in later stages of the cell cycle were progressively extracted for BM28, but staining for the RPA subunits did not diminish in intensity, and zones that stained for residual BM28 do not appear to be enhanced for RPA staining. Thus BM28 does not appear to concentrate in sites of active replication and behaves quite differently from RPA in terms of susceptibility to detergent extraction.

Comparing the level of BM28 in cell extracts with baculovirally expressed BM28 of known concentration, we estimate that there are ~10^5 molecules of protein per cell (data not shown). Thus, BM28 is an abundant protein, present at a level similar to that reported for other proteins thought to take part in chromatin structure, such as topoisomerase II (Gasser et al., 1986), matrix/scaffold at-
Discussion

Our initial immunofluorescence studies indicated that the BM28 protein is nuclear throughout interphase, suggesting that the changes in nuclear localization shown by Mcm2p and related proteins in *S. cerevisiae* (Hennessy et al., 1990; Yan et al., 1993) do not represent a conserved phenomenon in eukaryotic cells. However, by permeabilizing cells with nonionic detergents, we find that BM28 shows a cell cycle dependence in its nuclear binding. From late mitosis until S phase, nuclei contain BM28 that is refractory to detergent extraction, but as S phase proceeds, BM28 binding is lost. Since BM28 remains bound to S phase nuclei if DNA replication is inhibited by hydroxyurea treatment, DNA synthesis per se may be required to displace the protein. The nonextractable form of BM28 appears to be bound to chromatin. In extracted G1 nuclei, the BM28 distribution is very similar to that of DNA, as judged by DAPI staining, and BM28 can be released by DNase I digestion. During S phase, the distribution of BM28 changes dramatically, no longer showing an obvious correlation.
with DAPI staining, and, as replication proceeds, residual BM28 binding is seen in perinucleolar and envelope regions. During S phase, we had previously shown that BM28 does not concentrate in replication foci (Todorov et al., 1994), visualized by BrdU incorporation (Nakamura et al., 1986; Fox et al., 1991). In extracted mid–S phase cells, where BM28 antigen shows a speckled distribution, a similar result is obtained, and we also fail to detect general colocalization with PCNA, which is an accessory subunit of DNA polymerase 6 (Bravo et al., 1987), and RPA. Thus, BM28 is not enriched in the subnuclear foci, which represent the sites of active DNA synthesis (Cook, 1991).

In addition to changes in extractability, we have discovered cell cycle changes in the electrophoretic mobility of BM28 that may be related to the variation in the ease with which the protein can be removed from nuclei. The transition from mitosis to G1 is accompanied by a very rapid alteration in BM28 from a fast- to a slow-migrating form, and simultaneously, most BM28 becomes nonextractable with detergent. From S phase, there is a steady conversion of the protein from the slow- to the fast-migrating form. Thus, there is a close correlation between mobility changes in BM28, which we take to indicate some form of posttranslational modification, and changes in its nuclear affinity. Although these observations do not establish a causal link, the obvious possibility that a putative posttranslation modification is responsible for changes in binding are supported by cell fractionation experiments, which indicate that the slow form of BM28 is less detergent extractable than the fast form. The difference between the fast and slow forms of BM28 appears to be due to the level of phosphorylation; the higher level of phosphorylation of the fast form could affect its ability to bind chromatin. We note that the behavior of BM28 during the cell cycle, in terms of its varying extractability and the appearance of distinct forms in mitosis and G1, could allow its use as a marker for cell cycle transitions using assays based on immunofluorescence and immunoblotting.

These results are interpreted in terms of the model shown in Fig. 11. At a stage in late mitosis, BM28 is rapidly converted from a fast to a slow form by partial dephosphorylation and at the same time binds to decondensing chromatin. During S phase, displacement of BM28 from chromatin occurs perhaps as a result of activation of replication origins or elongation of replication forks. Displaced BM28 can diffuse out if the nuclear membrane is permeabilized by detergent, but in living cells the protein does not accumulate in the cytoplasm. This displacement from chromatin may require, or allow, conversion of BM28 to the fast form by hyperphosphorylation. Displaced protein may be incapable of rebinding to chromatin in S phase, perhaps due to inappropriate posttranslational modifications, or binding may be dependent on other factors available only during an “assembly” window during late mitosis. Finally, during mitosis, all BM28 is converted into the soluble fast form and remains extrachromosomal until telophase.

The recent finding that there appears to be a default pathway of export for nuclear proteins that are not bound to some nuclear structure (Schmidt-Zachmann et al., 1993) requires some comment. We suggest that BM28 may not be subjected to default export pathway either because it is too large (perhaps complexed with other proteins) or export does occur, but constitutive rapid import prevents cytoplasmic accumulation.

Overall, the behavior of BM28 is thus not so different to that shown by Mcm2p and related proteins in yeast, although the MCM proteins disappear from the nucleus after DNA replication, either as a result of export or degradation (Hennessy et al., 1990; Yan et al., 1993). Also, unlike BM28, the yeast proteins can be detected in the cytoplasm during interphase. This may well indicate that nuclear transport of the MCM proteins is restricted to a discrete phase of the cell cycle, as has been shown for Swi5p (Moll et al., 1991), but alternatively, the proteins could constantly shuttle between cytoplasm and nucleus and only be retained during the interval from mitosis to S phase. If both BM28 and the MCM proteins shuttle between cytoplasm and nucleus throughout the cell cycle, then the reason why human but not yeast nuclei retain the protein after DNA replication could simply reflect differences in the relative rates of nuclear import and export.

The cell cycle change in nuclear association of BM28 is broadly similar to that of murine P1, very recently reported by Kimura et al. (1994). BM28 and P1 are distinct members of the MCM2-3-5 family, P1 being most closely related to MCM5p, and although both MCM2 and MCM3 genes are essential, there is evidence for genetic interactions between them (Yan et al., 1991). Like BM28, P1 is a phosphoprotein, and the displaced form of the protein appears to be hyperphosphorylated compared with the bound form. One clear distinction between the behavior of the two proteins is that P1 does not appear to be chromatin associated; perhaps different members of the MCM2-3-5 family are associated with different subnuclear domains?

**Replication-mediated Changes in Chromatin and Cell Cycle Control**

Both BM28 and its yeast relatives appear to be necessary for normal DNA replication, suggesting that the presence of BM28 on chromatin may be necessary as a preparation for S phase. Onset of DNA synthesis at the G1–S transition requires an additional signal, probably involving activation of cyclin-dependent kinases (Roberts, 1993). Since
BM28 does not colocalize with sites of active DNA replication, we speculate that BM28 permits replication, either by allowing initiation or passage of a replication fork, but that it does not remain associated with either the replicome or the replicated DNA. Thus, during S phase, the residual BM28 staining seen in extracted nuclei could correspond to unreplicated DNA. Conceivably, the removal of BM28 from chromatin during replication could be relevant to the mechanism that limits DNA replication to one round per S phase. Thus the disassembly of BM28-containing complexes on replicated DNA during S phase, coupled with an inability to reassociate BM28 with chromatin until the following telophase, could prevent more than one round of DNA replication. The function of bound BM28 could thus be to “flag” unreplicated DNA. Studies of the S. cerevisiae origin-binding origin recognition complex have been interpreted to suggest a similar idea, namely, that in late anaphase or early telophase, a prereplicative complex may be assembled at replication origins, consisting of the origin recognition complex and another component, that permits initiation during the later S phase and disassembles after replication (Diffley et al., 1994).

In this context, comparisons can be drawn between the properties predicted for licensing factor and those of BM28. Thus, like licensing factor, BM28 could distinguish G1 from G2 chromatin, be required for replication, and may be in some way inactivated by passage of a replication fork. However, we have no evidence that the nuclear membrane is important for regulating BM28 function, and in any case, there is no direct evidence that BM28, or its yeast relatives function as regulatory rather than as necessary replication components.

The implication that G1 and G2 chromatin have different protein compositions could have a relevance to processes other than DNA replication. For instance, the restriction of transcription to particular phases of the cell cycle could depend, in part, on transcription factors bound to the chromatin template that are displaced by replication and cannot rebind until the following G1 phase (Rita et al., 1991). This would restrict their period of activity to a discrete portion of interphase. In addition, the surveillance mechanism that prevents mitosis if S phase is incomplete (Hartwell and Weinert, 1989; Enoch and Nurse, 1990) could function in part by monitoring proteins that are found on G1 but not G2 chromatin. If proteins associated with G1 chromatin, normally displaced by replication, generated a trans-acting signal that blocked the activation of the p34 kinase responsible for the G2 to M transition, this would provide a device to link mitosis to the completion of DNA synthesis.

We thank P. Cook, C. Graham, and D. Maiorano for comments on the manuscript. We are grateful to A. W. Alberts for the generous gift of lovastatin and advice on its use, J. Hurwitz for the anti-RPA antibodies, and D. Shotton for the use of his microscope facility.

This work was supported by grants from the Cancer Research Campaign and the European Commission. A. Attaran was supported by a Howard Hughes Medical Institute Predoctoral Fellowship.

Received for publication 19 October 1994 and in revised form 27 January 1995.

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