A murine replication protein accumulates temporarily in the heterochromatic regions of nuclei prior to initiation of DNA replication

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SUMMARY

We have analyzed the expression of the murine P1 gene, the mammalian homologue of the yeast MCM3 protein, during the mitotic cell cycle. The MCM3 protein has previously been shown to be of importance for initiation of DNA replication in Saccharomyces cerevisiae. We found that the murine P1 protein was present in the nuclei of mammalian cells throughout interphase of the cell cycle. This is in contrast to the MCM3 protein, which is located in the nuclei of yeast cells only between the M and the S phase of the cell cycle. Detailed analysis of the intranuclear localization of the P1 protein during the cell cycle revealed that it accumulates transiently in the heterochromatic regions towards the end of G1. The accumulation of the P1 protein in the heterochromatic regions prior to activation of DNA replication suggests that the mammalian P1 protein is also of importance for initiation of DNA replication. The MCM2-3-5 proteins have been suggested to represent yeast equivalents of a hypothetical replication licensing factor initially described in Xenopus. Our data support this model and indicate that the murine P1 protein could function as replication licensing factor. The chromosomal localization of the P1 gene was determined by fluorescence in situ hybridization to region 6p12 in human metaphase chromosomes.

Key words: DNA replication, cell cycle, mitosis, licensing factor, MCM2-3-5 family

INTRODUCTION

Initiation of DNA replication in eukaryotes is a carefully regulated process, involving both activation mechanisms that promote initiation of DNA replication within one S phase, and repression mechanisms that prevent a second round of initiation within the same S phase, as well as at other stages of the cell cycle (reviewed by Fangman and Brewer, 1992; DePamphilis, 1993). In order to understand how this temporal activation of individual replication origins is achieved, it will be important to define the proteins that bind to and activate them.

A group of sequences, called autonomously replicating sequences (ARS), have been identified by their ability to mediate extrachromosomal replication in yeast (Brewer and Fangman, 1987; Huberman et al., 1987). A protein complex, the origin recognition complex (ORC) interacts with ARS in yeast, both in vitro and in vivo (Bell and Stillman, 1992; Diffley and Cocker, 1992). Genes encoding two different components of this complex, ORC2 and ORC6, have been cloned and characterized (Bell et al., 1993; Foss et al., 1993; Li and Herskowitz, 1993; Micklem et al., 1993). The origin recognition complex is most likely bound to replication origins throughout the cell cycle (Diffley and Cocker, 1992), indicating that in order to initiate DNA replication at the appropriate time point during S phase, additional proteins interacting with the ORC are required. Interestingly, the ORC6 protein has been shown to interact with a protein known to be essential for the early steps of DNA replication in Saccharomyces cerevisiae, CDC46/MCM5 (Li and Herskowitz, 1993).

The CDC46/MCM5 gene is a member of the MCM2-3-5 gene family, including at least two other genes, MCM2 and MCM3 (Gibson et al., 1990; Hennessy et al., 1990, 1991; Yan et al., 1991; Chen et al., 1992; Tye, 1994). Mcm mutants have been shown to be defective in minichromosome maintenance in an ARS-specific manner, to have a reduced capacity for initiating DNA synthesis at chromosomal replication origins, and to exhibit a premiotic cell cycle arrest as well as an increase in chromosome loss and recombination (Gibson et al., 1990; Yan et al., 1991, 1993; Chen et al., 1992). Although these proteins are structurally related, they cannot functionally substitute for one another (Gibson et al., 1990; Hennessy et al., 1991; Yan et al., 1991). Taken together, these observations suggest that the MCM2-3-5 family of proteins in S. cerevisiae have an essential function during the early stages of DNA replication, possibly regulating initiation of DNA replication during G1 (reviewed by Tye, 1994).

The most suggestive evidence that the MCM2-3-5 proteins play a role in initiation of DNA replication comes from an analysis of their intracellular distribution during the cell cycle.
The subcellular localization of these proteins is regulated in a cell cycle-dependent manner, i.e. these proteins will enter the nucleus at the end of mitosis and disappear from it at the G1/S boundary (Hennessy et al., 1990; Yan et al., 1993; Tye, 1994). This expression pattern coincides with that of a hypothetical replication licensing factor in higher eukaryotes, a factor suggested to be involved in regulation of initiation of DNA replication (Blow and Laskey, 1988; Blow, 1993; Coverley et al., 1993).

Genes related to the MCM2-3-5 gene family in S. cerevisiae have been isolated in other organisms also, including Schizosaccharomyces pombe (Coxon et al., 1992; Miyake et al. 1993) and mammals (Thömmes et al., 1992; Hu et al., 1993; Todorov et al., 1994). Microinjection of antibodies directed against the BM28 protein, the human homologue of the MCM2 protein, inhibits initiation of DNA replication in mammalian cells, whereas it has no effect on DNA elongation (Todorov et al., 1994). The human homologue of the MCM3 gene, P1, encodes a 105 kDa nuclear protein loosely associated with the replication-specific DNA polymerase α/primase (Thömmes et al., 1992).

We have studied the expression pattern of the murine homologue of the MCM3 protein (P1), in order to better understand the function of this protein. The P1 protein, in contrast to the yeast MCM3 protein, was found to be localized in the nuclei of cells throughout interphase of the cell cycle. Analysis of the intranuclear localization of the P1 protein revealed that it accumulated transiently in the heterochromatic regions during the G1 phase of the cell cycle. This indicates that the mammalian P1 protein is involved in a nuclear activity occurring prior to activation of DNA replication.

**MATERIALS AND METHODS**

**Isolation of the TSG23 cDNA clone**

A cDNA clone, TSG23, was isolated from an oligo(dT)-primed cDNA library as part of a screen for cDNA clones predominantly expressed in juvenile mouse testis (Starborg et al., 1992). TSG23 contained a 2039 bp long insert representing the 3′ end of the murine P1 cDNA clone (Thömmes et al., 1992).

**Preparation of antibodies**

A 913 bp fragment, position 1317-2201 in the murine P1 cDNA sequence (Thömmes et al., 1992), was amplified from the TSG23 cDNA clone using the polymerase chain reaction technique. The 5′ end primer had the sequence 5′-ACCGTTGGATCCGGCAGGTAT-GATCGTATAAAG-3′, whereas the sequence of the 3′ end primer was 5′-TCAGTAAGCTT CCTCTTAGAAGTGTTCCAGG-3′. The two oligonucleotides that were used contained, in addition to a sequence complementary to the desired location in the murine P1 cDNA clone (underlined), additional bases including restriction sites for the enzymes BamHI (5′ end primer) and HindIII (3′ end primer). The amplified fragment was, after digestion with BamHI and HindIII, ligated in-frame to the murine DHFR gene, and as well as to an 18 base sequence encoding six histidine residues, included in the bacterial expression vector pQE-13 (Qiagen). The resulting 23.EXPR plasmid was transformed into M15pREP4 Escherichia coli cells. Transformed cells were grown in large cultures and the production of the recombinant protein was induced by IPTG. The induced cells were lysed by addition of guanidinium-HCl and the histidine-tagged recombinant protein was purified in one step by Ni2+-chelate affinity chromatography, as described by the supplier (Qiagen). The eluted recombinant protein was dialyzed against PBS buffer. Female New Zealand rabbits were injected intradermally with 600 µg of the bacterially expressed murine P1 protein, in Freund’s incomplete adjuvant, and boosted every 4 weeks with 250 µg of the same protein preparation in Freund’s incomplete adjuvant, injected subcutaneously, and bled 10 days after each booster.

**Affinity purification of polyclonal antibodies against the P1 protein**

The polyclonal antibodies raised against the murine P1 protein were affinity-purified according to Sambrook et al. (1989). Briefly, after purification on a Ni2+-chelate affinity column, the bacterially produced TSG23 recombinant protein was subjected to gel electrophoresis on a 12% SDS-PAGE gel. The protein was transferred electrophoretically to a nitrocellulose filter and a strip was cut out from the middle part of the filter and stained with Comassie Brilliant Blue. The stained strip was put back between the two halves of the filter and a strip corresponding to the protein band was cut out. The strip was put into a Petri dish, blocked with 3% BSA for 1 hour at room temperature and then incubated with either total TSG23 rabbit serum or preimmune serum, diluted 1:10 in blocking buffer, and incubated overnight at 4°C. The strip was washed at room temperature for 20 minutes with 0.15 M NaCl and 20 minutes with PBS, cut into smaller pieces, transferred to an Eppendorf tube and incubated with 0.2 M glycine, pH 2.8, 1 mM EGTA. The eluted fractions were neutralized to pH 7 with 1 M Tris-HCl (Sambrook et al., 1989).

**Immunoblotting**

Mouse Swiss 3T3 fibroblasts were washed with PBS and lysed with SDS-reducing buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 10 mM DTT and 4 M urea) as described by Harlow and Lane (1988). Extracts were separated on 7.5% SDS-PAGE and proteins were transferred to an Immobilon-P filter in transfer buffer (41 mM Tris, 192 mM glycine, 0.02% SDS, pH 8.3; Laemmli, 1970). The filters were blocked with 3% BSA in PBS with 0.01% Tween-20 for 1 hour at room temperature, then incubated with anti-P1 antibodies (diluted 1:200), or a pre-immune serum-anti-peptide in a similar way (diluted 1:200), for 1 hour at room temperature. After washes in PBS containing 0.05% Tween-20, a horseradish peroxidase-conjugated anti-rabbit antibody (Dakopatts P399) was added at a dilution of 1:3000, and incubated for 40 minutes at room temperature. Detection of the immune signal was performed using the ECL western blotting detection system (Amersham Corp.).

**Cell culture and synchronization**

Mouse Swiss-3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DME), from Gibco, containing 10% fetal calf serum (Sigma). Cells were plated at low density and grown at 37°C in a humidified atmosphere containing 5% CO2, 95% air. To synchronize cells, 3T3 cells were cultured in DME medium, including 0.1% fetal calf serum (Sigma), for 48 hours (Braun and Macdonald-Braun, 1987; Fox et al., 1991; Björklund et al., 1992). An equal number of cells (1x10^6) were then processed at 2 hour intervals following addition of DME medium, including 10% fetal calf serum. The synchrony of the cell population at each 2 hour interval were determined by incorporation of BrdU for 30 minutes prior to fixation of the cells. The cells were then subsequently stained with an anti-BrdU antibody (Amersham Corp.). The fraction of BrdU positive cells was determined at different time points and the percentage BrdU positive cells was calculated: 0 hour, 10%; 2 hours, 5%; 4 hours, 5%; 6 hours, 3%; 8 hours, 7%; 10 hours, 3%; 12 hours, 45%; 14 hours, 49%; 16 hours, 58%; 18 hours, 65%; 20 hours, 40%; and 22 hours, 29%. In accordance with other studies (Björklund et al., 1992; Todorov et al., 1994), we found that approximately 50% of the Swiss-3T3 cells initiated S phase 12 hours after the addition of serum, and that the S phase lasted for 6-8 hours, i.e. the G2 phase was initiated approximately 20-22 hours after serum addition. Samples for immunoblotting were
dissolved in SDS-reducing buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, and 10 mM DTT) as described by Harlow and Lane (1988). Immunoblotting was performed as described above. The amount of protein used for each time point was analyzed by immunoblotting with an anti-α-lamin antibody (diluted 1:1000, a gift from Dr Spyrou Georgatos, EMBL, Heidelberg), or by staining the filters with Coomassie Blue (R 250).

**Immunofluorescence**

Swiss-3T3 cells were cultured at low density on coverslips. Cells synchronized as described above, as well as asynchronously growing cells, were fixed in ice-cold methanol:acetone (50:50, v/v) for 5 minutes and pre-incubated with 3% BSA prior to addition of the first antibody. The affinity-purified murine anti-P1 antibody was diluted 1:20, and the monoclonal anti-PCNA antibody 1:200 (Boehringer Mannheim). The secondary antibodies were fluorescein isothiocyanate-conjugated swine anti-rabbit IgG (diluted 1:50, Boehringer Mannheim), and rhodamine-conjugated goat anti-mouse IgG (diluted 1:50, Boehringer Mannheim). The cells were stained with 1 µg/ml of Hoechst 33258 for 1 minute. The slides were mounted in a 78% glycerol mounting medium, containing 1 mg/ml para-phenylene diamine, examined in a Nikon Labophot microscope and photographed with Kodak T-Max 400 film.

**Analysis of the chromosomal localization**

A human placenta cosmid library in the vector pWE15 (Evans et al., 1989), was screened with a 900 bp fragment derived from the 3′ end of the TSG23 cDNA clone (position 1910-2813 of the murine P1 cDNA sequence), and two different clones were isolated (Sambrook et al., 1989). To confirm the identity of the cosmids, two oligonucleotides derived from the murine P1 cDNA sequence, 5′-GGCAGGTATGATCAGTATAA-3′, position 1318-1337, and 3′-GATGTACCCTGGTTCTTCTT-5′, position 1603-1623, were used for PCR amplification (Saiki et al., 1988). The amplified DNA fragments were purified from low-melting agarose and sequenced directly using fluorescently labelled deoxy-terminators, as described by the manufacturer (Applied-Biosystems). Slides from human metaphase chromosomes were prepared from standard lymphocyte cultures from normal, healthy individuals. The slides were postfixed, RNase-treated and denatured as previously described (Pinkel et al., 1986; Blennow et al., 1992). The two human cosmids clones isolated with the TSG23 probe were used as probes after labelling with biotin-16-dUTP (BRL). The probe (50 ng) was preannealed with Cot-1 DNA (4-8 µg) for 60 minutes at 37° C. Hybridization was performed in 50% formamide, 2× SSC at 42° C overnight. The slides were washed three times for 5 minutes in 50% formamide, 2× SSC at 42° C, and three times in 0.1× SSC at 60° C. After washing, the hybridized probe was coupled to fluorescein-isothiocyanate-avidin D. The chromosomes were counterstained with propidium iodide, and the results were analyzed and photographed using confocal laser scanning microscopes (Zeiss and Leica). The chromosomal localization of the signal was identified by quinacrine (QFQ) banding. High molecular mass DNA was isolated from hamster/human somatic hybrid cell lines retaining various individual human chromosomes in addition to the rodent genomes (NIGMS Coriell Cell Repositories), digested with HindIII, size-fractionated in 0.9% agarose gels, transferred to nylon membrane and hybridized with [α-32P]dCTP-labelled cosmid DNA, as previously described (Bergerheim et al., 1989).

**RESULTS**

**Molecular cloning and mapping of the P1 gene**

We have previously isolated a cDNA clone (TSG23), from a juvenile mouse testis cDNA library using a differential screening procedure, and shown that this cDNA clone identifies a mRNA band that is strongly expressed in juvenile testis (Höög, 1991; Starborg et al., 1992). Initially, no sequences similar to TSG23 were found in the EMBL data library. A more recent comparison, however, of the sequence of the TSG23 cDNA clone with the content of the EMBL data library, revealed that the sequence of this clone was identical with region 774 to 2813 of the murine P1 gene (Thömmes et al., 1992).

The localization of the P1 gene on human chromosomes was investigated by fluorescence in situ hybridization (FISH). A human genomic cosmid library was screened with the murine P1 cDNA clone and two different cosmid clones were isolated. The identities of these cosmids clones were confirmed by PCR amplification, using oligonucleotides derived from the murine P1 cDNA sequence, and DNA sequencing of the amplified DNA fragments (see Materials and Methods). The two human P1-containing cosmid clones were labelled with biotin-16-dUTP and hybridized to human metaphase chromosomes. The hybridizing signal was detected as asymmetrical spots on both chromatids next to the centromere of chromosome 6, and by QFQ banding this signal was further mapped to the 6p12 region (Fig. 1). No additional hybridization signal was seen. The chromosomal localization of P1 gene was further corrob-
on a rodent background (not shown). The P1 probe detected a 
somatic cell hybrids retaining individual human chromosomes
orated after hybridization of the murine P1 cDNA clone to 
given in kilodaltons.

(Fig. 2). or an affinity-purified anti-DHFR antibody. Molecular sizes are 
gel electrophoresis. After immunoblotting, the filters were analyzed 
(A,B,C) and the proteins were separated using SDS-polyacrylamide

Characterization of the P1 product

In order to analyze the expression and function of the murine 
P1 protein during the mitotic cell cycle, we developed a poly-
clonal antibody serum against the P1 protein. A 200 amino acid
region from the 3′ end of the P1 cDNA sequence was overex-
pressed in bacteria, purified, and injected into rabbits (see 
Materials and Methods). This region of the P1 protein has been 
shown not to be conserved in the different members of this gene
family (Thömmes et al., 1992; Hu et al., 1993; Todorov et al., 
1994). The rabbit sera were affinity-purified, and the affinity-
purified polyclonal sera were used to analyze protein extracts 
prepared from Swiss-3T3 cells. A protein with a molecular
mass of approximately 105 kDa was visualized with the anti-
P1 antibody (Fig. 2). A protein band with a similar molecular
mass was also observed in protein extracts prepared from HeLa 
cells, suggesting that the murine anti-P1 antibody also reacts 
with the human P1 protein (data not shown). These results are 
very similar to the findings of Thömmes et al. (1992), who iden-
tified a 105 kDa human protein in extracts prepared from HeLa 
cells and calf thymus. No additional bands of higher or lower
molecular sizes were observed in the murine extracts, suggest-
ing that the anti-P1 antibody does not crossreact with other
members of this family, e.g. the BM28 protein, which migrates 
as a 125 kDa band on SDS-PAGE gels (Todorov et al., 1994).

Cell cycle expression and cellular localization of the 
P1 protein

We used the affinity-purified anti-P1 antibody to investigate 
the expression of the P1 protein in mammalian cells. The
antibody was applied to methanol/acetone-fixed asynchronously 
growing Swiss/3T3 cells and the result was analyzed by 
indirect immunofluorescence microscopy. The same cells 
were also stained in parallel, with Hoechst 33258, a DNA 

binding dye that preferentially binds to the intranuclear heter-
ochromatic regions of murine cells (see e.g. Nakayasu and 
Berezney, 1989). The anti-P1 antibody was found to label the 
nuclei uniformly, with the exception of the nucleoli and the 
heterochromatic regions (not shown). A detailed comparison 
of the intranuclear distribution of the P1 protein in different 
cells, however, revealed that the relative size of the regions not 
labelled by the anti-P1 antibody varied considerably. Further-
more, in a few cells the P1 pattern was found to overlap with 
the regions of the nuclei labelled by Hoechst 33258.

In order to investigate if the different intranuclear patterns 
displayed by the anti-P1 antibody could be linked to the pro-
gression of the cell cycle, as well as to further ensure that the 
P1 protein was present in the nuclei of cells throughout inter-
phase of the cell cycle, the expression of the P1 protein in 
serum-starved cells was analyzed. Swiss-3T3 cells were syn-
chronized in G0 by serum starvation for 2 days, after which 
they were given medium containing 10% serum, in order to 
reinitiate the cell cycle. The synchronization of the cells was evaluated 
by monitoring the incorporation of bromodeoxyuridine at 
different time points (see Materials and Methods). S phase was 
found to be initiated 12 hours after the addition of serum, and 
lasted for 6-8 hours, i.e. the G2 phase was initiated approxi-
ately 20-22 hours after serum addition.

The synchronously growing Swiss-3T3 cells were analyzed 
in parallel using the affinity-purified anti-P1 polyclonal antibody, 
a monoclonal antibody directed against PCNA (the 
proliferating cell nuclear antigen), and by staining of the cells 
with Hoechst 33258. The P1 was found to be localized in the 
nuclei of all interphase cells (Fig. 3A, D1, D2, G, J, M), and 
in the extrachromosomal region of mitotic cells (Fig. 3P). A 
comparison of the intranuclear distribution of the P1 protein 
revealed a similar, but not identical, pattern in cells 5 hours 
(Fig. 3A) 12 hours (Fig. 3G), 18 hours (Fig. 3J) and 22 hours 
(Fig. 3M) after serum addition, i.e. in early G1, early S, late S 
and G2 cells, respectively. It was apparent that the intranuclear 
regions not labelled by the anti-P1 antibody were larger in cells 
that had reached the G2 phase of the cell cycle, compared to 
cells at earlier stages of the cell cycle (Fig. 3M). A compar-
ison of the P1 pattern with the pattern seen after staining with 
Hoechst 33258 revealed that in most cells these two patterns 
were not overlapping (Fig. 3). However, 10 hours after serum 
addition (late G1) a different intranuclear pattern was visible 
in the majority of the cells. In these cells, the anti-P1 antibody 
gave rise to bright foci against a background of diffuse nuclear 
labelling (Fig. 3D1, D2), a pattern that was seen only in a 
minority of the cells before or after this time point. A com-
parison with Hoechst 33258 labelling, showed that the bright 
focus formed in the nuclei 10 hours after serum addition, colo-
ralized with the foci formed by Hoechst 33258 (compare Fig. 
3D1 with F1, and Fig. 3D2 with F2). As Hoechst 33258 pre-
viously has been shown to preferentially stain heterochromatic 
regions in murine cells, this result suggests that the P1 protein 
accumulates transiently in the heterochromatic regions of the 
nucleus.

To clearly establish at what time point during the cell cycle 
the P1 protein begins to accumulate in the heterochromatic 
region, it was necessary to include a marker for DNA replic-
tion. It has been shown that euchromatic and heterochromatic 
DNA are being replicated at different time points during S 
phase, a process which can be monitored using antibodies

![Fig. 2. Characterization of the anti-P1 antibodies. Protein extracts were prepared from asynchronously growing Swiss/3T3 cells (A,B,C) and the proteins were separated using SDS-polyacrylamide gel electrophoresis. After immunoblotting, the filters were analyzed using the affinity-purified anti-P1 antibody (A), a pre-immune serum (B), or an affinity-purified anti-DHFR antibody. Molecular sizes are given in kilodaltons.](Image)
Expression of the murine P1 protein directed against PCNA (a subunit of DNA polymerase δ), or by direct incorporation of BrdU into newly replicated chromosomal DNA, two methods that give very similar results (Celis and Celis, 1985; Bravo and Macdonald-Bravo, 1987; Nakayasu and Berezney, 1989; Fox et al., 1991; Humbert et al., 1992). We therefore compared the pattern displayed by the anti-P1 antibody with the pattern seen with the anti-PCNA antibody (Fig. 3D1-E1, D2-E2, G-H). Replication granules (as displayed by the anti-PCNA antibody), were first seen in the synchronized cells 12 hours after addition of serum (Fig. 3H), suggesting that a majority of the cells at this time point have reached early S phase. The P1 protein, however, accumulated in the heterochromatic regions of the nuclei prior to this time point (Fig. 3D1, D2). A comparison of the P1 pattern with the pattern seen after incorporation of BrdU into chromosomal DNA gave similar results, i.e. the accumulation of the P1 protein in the heterochromatic regions always occurred prior to the formation of replication granules (data not shown).

Fig. 3. Cell cycle expression and localization of the P1 protein in synchronized mouse Swiss/3T3 cells. Cells were synchronized as described in Materials and Methods, and analyzed at different time points after addition of 10% serum to the cells: 5 hours (A-C), 10 hours (D-F), 12 hours (G-I), 18 hours (J-L), 22 hours (M-O). Cells at different time points were stained in parallel with the affinity-purified anti-P1 antibody (A,D,G,J,M), a monoclonal anti-PCNA antibody (B,E,H,K,N), and Hoechst 33258 (C,F,I,L,O). The secondary antibodies used were a FITC-conjugated swine anti-rabbit IgG and a rhodamine-conjugated goat anti-mouse IgG, after which the cells were analyzed using indirect immunofluorescence microscopy. A cell undergoing mitosis was investigated in a similar way with the anti-P1 antibody (P), the anti-PCNA antibody (Q), and stained with Hoechst 33258 (R). As negative controls, cell were stained with a pre-immune serum (S), an anti-DHFR antibody (U), or stained with Hoechst 33258 (T, V). Bar, 20 µm.
The anti-P1 antibody pattern was also compared with the anti-PCNA pattern 18 hours after serum addition. At this time point the anti-PCNA antibody gave rise to bright foci in the nuclei of cells, a pattern typical of late S phase cells (Celis and Celis 1985; Bravo and Macdonald-Bravo, 1987; Nakayasu and Berezney, 1989; Fox et al., 1991; Humbert et al., 1992), i.e. in these cells the heterochromatic regions, which can be stained in parallel with Hoechst 33258, are being replicated (Fig. 3KL). At this stage of the cell cycle, however, we have not been able to detect the same type of bright P1 foci that were seen 10 hours after serum addition. In summary, these results strongly suggest that the P1 protein accumulates temporarily in the heterochromatic regions of the nuclei, and that this accumulation process occurs prior to the formation of replication granules.

Indirect immunofluorescence microscopy represents only one technique which can be used to investigate the presence of a protein in a cell. Therefore, the total level of the P1 protein in synchronized cells was also analyzed using immunoblotting methods. Protein samples were prepared from cells 5 hours, 10 hours, 12 hours, 18 hours and 22 hours after addition of serum to the arrested cells, and analyzed using the anti-P1 antibody, as well as with an anti-α-lamin antibody, to calibrate for the amount of protein loaded in each lane (Fig. 4). The amount of P1 protein relative to α-lamin was found to be rather constant, although a small reduction of the amount of P1 protein at 22 hours after serum addition was apparent. The result of this experiment suggests that the total level of P1 protein is relatively constant throughout the cell cycle.

DISCUSSION

We have analyzed the expression of the murine P1 protein, a homologue of the *S. cerevisiae* MCM3 protein, in order to further define the function of this protein. We find that the P1 protein is present in the nuclei of the Swiss/3T3 cells throughout interphase, and that the amount of P1 protein appears to be rather uniform throughout the cell cycle (Fig. 3). This is an interesting observation, as it was previously shown that the yeast MCM2-3-5 proteins are present in the nuclei of cells only transiently, i.e. these proteins will enter the nucleus at the end of mitosis and disappear from it at the G1/S boundary (Hennessy et al., 1990; Yan et al., 1993; Tye, 1994). The BM28 protein, a human homologue of the MCM2 protein, was recently shown to be localized in the nuclei of HeLa and NIH3T3 cells (Todorov et al., 1994), and Thömmes et al. (1992) have shown that the human P1 protein is localized in the nuclei of proliferating Hel 299 cells. Taken together, these results indicate that, in contrast to the MCM2 and the MCM3 proteins in *S. cerevisiae*, the homologous proteins in mammals are present in the nuclei throughout the cell cycle. Immunoblotting analysis of the level of the P1 protein in synchronized cells revealed that the total amount of this protein did not change considerably throughout interphase, supporting the results of the immunoinduction experiments (Fig. 4).

Immunoblotting analysis of the CDC46/MCM5 protein has shown that the total level of this protein also is relatively constant throughout interphase, suggesting that the disappearance of the CDC46/MCM5 protein from the nuclei of S and G2 phase cells, as shown by indirect immunofluorescence microscopy, is compensated by an increased level of this protein in the cytoplasm of yeast cells (Hennessy et al., 1990). Homologues of the MCM family of proteins have been identified in *S. pombe* also (Coxon et al., 1992; Miyake et al., 1993), and future experiments with this organism will establish if these proteins are present in the nuclei of cells throughout interphase, or if they undergo subcellular fluctuations similar to those described for this group of proteins in *S. cerevisiae*.

When the intranuclear localization of the P1 protein was analyzed in synchronized cells, it was found that the anti-P1 antibody gave rise to bright foci in cells 10 hours after addition of serum (Fig. 3). These foci were seen only transiently, strongly suggesting that their formation was cell cycle-regulated. The DNA replication process was monitored in parallel in the synchronized cells using anti-PCNA antibodies or BrdU incorporation, and it was found that the formation of the P1 foci preceded the formation of replication granules (Fig. 3). The experimental data concerning the yeast MCM protein family suggest that this group of proteins execute their functions during the G1 phase of the cell cycle (Gibson et al., 1990; Hennessy et al., 1990, 1991; Yan et al., 1991, 1993; Chen et al., 1992). Furthermore, microinjection of antibodies directed against the BM28 protein during G1 was found to inhibit DNA replication, whereas no effect was seen on DNA elongation, when the same antibodies were injected during S phase (Todorov et al., 1994). The assembly of P1 proteins into bright foci prior to initiation of DNA replication, as shown here, gives further support to the model that this family of proteins is involved in a nuclear activity that takes place during the G1 phase of the cell cycle.

The bright foci, seen with the anti-P1 antibody, were found to colocalize with regions of the nuclei that were preferentially stained with Hoechst 33258 (Fig. 3), suggesting that the P1 protein accumulates in the heterochromatic regions of the nuclei. This result indicates that there exist at least two different forms of the P1 protein in the nuclei of interphase cells, one form that specifically becomes associated with chromatin at the end of the G1 phase of the cell cycle, and another form that gives rise to the diffuse nuclear labeling that is seen with the anti-P1 antibody throughout interphase. Similar conclusions have been reached also by cellular frac-
tion experiments, i.e. a fraction of the human P1 protein is bound to a nuclear structure (Thömmes et al., 1992) and, likewise, a fraction of the MCM3 protein is bound to chromatin during the G1 phase of the cell cycle (Yan et al., 1993).

A central question now is of course to identify the components of chromatin with which this protein family is interacting. It was recently shown that the CDC46/MCM5 protein interacts with the ORC (Li and Herskowitz, 1993), a protein complex that is bound to replication origins throughout the cell cycle (Difffley and Cocker, 1992). Furthermore, the mammalian P1 protein is loosely associated with the holoenzyme form of the DNA polymerase α/primase complex, a multiple-protein complex consisting of 10-16 different protein subunits (Thömmes et al., 1992). This protein complex has been shown to be present in the nuclei of mammalian cells during G1 (Thömmes et al., 1992; Nakamura et al., 1984). Together, these data indicate that the MCM2-3-5 family in both yeast and mammals associates with a chromatin component, possibly with a prereplication complex, prior to initiation of DNA replication. Sequence comparisons have revealed that the MCM2-3-5 family belongs to a superfamilly of DNA-dependent ATPases, suggesting that they mediate ATP-dependent opening of double-stranded DNA in the replication origins (Koonin, 1993). We have been able to observe an accumulation of the P1 protein only in the heterochromatic regions of the nuclei; however, it is likely that a similar accumulation of the P1 protein also occurs in the euchromatic regions. In contrast to the heterochromatic regions, the rest of the nucleus is strongly stained by the anti-P1 antibody throughout G1 phase, making it technically difficult to detect a temporally restricted association of the P1 protein with the euchromatic regions.

It has been suggested that the MCM2-3-5 family of proteins represent the yeast equivalents of a hypothetical replication licensing factor, initially described in Xenopus (Hennessy et al., 1990; Yan et al., 1993; Tye, 1994). The licensing factor is believed to be able to enter the nucleus only during mitosis, to directly modify chromosomes, to be required for initiation of DNA replication, and to support only one round of DNA replication during each cycle (Blow and Laskey, 1988; Blow, 1993; Coverley et al., 1993). The replication licensing factor becomes activated after completing metaphase, but prior to formation of an intact G1 nucleus. The activation process is dependent on a protein kinase, suggesting that it is the result of a post-transcriptional event (Blow, 1993). If the P1 protein is identical to the replication licensing factor it appears that it has to undergo at least two independent post-transcriptional regulatory events: one activation event occurring during mitosis, necessary for its interaction with replication origins, and one inactivation event occurring at the G1/S transition, prohibiting further activation of DNA replication. In accordance with this model, we find a high level of the P1 protein in mitotic cells and, once G1 is initiated, that the P1 protein becomes localized in the nucleus. The P1 protein is found to transiently interact with chromatin prior to initiation of DNA replication, and is subsequently lost from chromatin at the G1/S border. The reason for the apparent delay between nuclear entry and chromatin association during G1 is not clear (Fig. 3), and could be an effect of the synchronization process that the cells have undergone. An alternative, and more interesting possibility, however, would be that the P1 protein is undergoing two separate activation processes: one taking place at mitosis during which the P1 protein becomes competent to initiate DNA replication, and a second taking place at the end of G1 during which the P1 protein becomes associated with the chromatin. Another replication protein, replication factor A (RPA), also fulfills many of the requirements for a replication licensing factor, included cell cycle-regulated phosphorylation (Din et al., 1990; Dutta and Stillman, 1992; Fotedar and Roberts 1992), and transient accumulation onto chromatin prior to initiation of DNA replication (Adachi and Laemmli, 1992). Further studies will be necessary in order to establish if the MCM2-3-5 proteins, or the RPA protein, function as a replication licensing factor.

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