Expression, phosphorylation and nuclear localization of the human P1 protein, a homologue of the yeast Mcm 3 replication protein

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SUMMARY

The human protein P1 belongs to a newly discovered class of mammalian nuclear proteins with high sequence homology to yeast replication proteins. We present the entire amino acid sequence of the human protein P1 as predicted from the cDNA sequence, and show that P1 shares three central regions of high sequence similarity (about 75%) and a highly hydrophilic carboxy-terminal region with the yeast Mcm3 replication protein. The human genome most probably contains one P1 gene which is activated when HeLa cells progress to S phase, as shown by a several-fold increase in P1-specific mRNA. However, the amounts of P1 protein do not detectably change during this period, but P1 protein becomes phosphorylated at the beginning of S phase. In contrast to the yeast Mcm proteins, which disappear from nuclei after initiation of DNA replication, protein P1 remains in the nucleus during and after S phase. P1 is dispersed in mitotic cells and may be excluded from binding to chromosomes.

Key words: Mcm protein, protein P1Mcm3, genome replication, gene locus

INTRODUCTION

The P1 protein is a member of a recently discovered family of mammalian nuclear proteins (Thömmes et al., 1992). These proteins are believed to function in genome replication because they share long stretches of amino acids with yeast replication proteins (Hu et al., 1993).

The homologous proteins in Saccharomyces cerevisiae are defined by mutants unable to sustain the replication of extrachromosomal DNA with autonomously replicating sequences (ARS), hence their designation as minichromosome maintenance or mcm mutants (Gibson et al., 1990; Yan et al., 1991). Work with S. cerevisiae led to the identification of three structurally related MCM genes that are required for replication, MCM 2, MCM 3 and MCM 5 (Chen et al., 1992). The MCM 5 gene is identical with a previously described yeast cell cycle arrest gene defined by the cdc46 mutant (Hennessy et al., 1990, 1991). The products of these yeast genes are proteins with high degrees of sequence similarity, showing that they belong to one protein family. Although the proteins are structurally and functionally related they cannot substitute for one another, indicating that they may perform related but independent functions (Yan et al., 1991).

Interestingly, the different mcm mutants affect the stability of minichromosomes to different degrees depending on the particular ARS element in the minichromosomes (Gibson et al., 1990). These findings suggest that different Mcm proteins interact differently with ARS elements. In addition, the frequency of replication initiation at chromosomal origins is also reduced in these mutants, and experiments suggest that Mcm proteins have an execution point at the beginning of the S phase, implying that they are somehow involved in the initiation of genome replication (Yan et al., 1993).

The yeast Mcm proteins have a characteristic cell-cycle-dependent variation in nuclear localization: the proteins enter the nucleus at the end of mitosis, remain in the nucleus throughout G₁ phase and become predominantly cytoplasmic at the G₁/S transition. This change in nuclear localization has suggested to some workers that the Mcm proteins may be related to the hypothetical licensing factor in genome replication (Hennessy et al., 1990; Yan et al., 1993). A licensing factor has been proposed by Blow and Laskey (1988) to explain the fact that re-replication of nuclear DNA requires a breakdown of the nuclear envelope; this would allow essential proteins to gain access to the nucleus where they bind to chromatin until they are consumed during the next S phase.

The yeast nuclear envelope does not break down during mitosis, and this is one important feature distinguishing the mitotic cycles of yeast and most metazoan eukaryotes. It is therefore of interest to investigate the cell-cycle-dependent expression and localization of protein P1, the mammalian counterpart of the yeast Mcm 3 protein (Hu et al., 1993).

We first determined the complete primary structure of human P1 protein by cDNA sequencing and identified the
chromosomal localization of its gene. We then investigated its expression and its subcellular distribution during the progression through the G1 phase and the S phase of the cell cycle. Using indirect immunofluorescence procedures, we found protein P1 in the nucleus during G1, S and G2 phases. But P1 appears to be excluded from mitotic chromosomes and distributed throughout the cell in mitosis. In addition, our results show that protein P1 is phosphorylated in a cell-cycle-dependent manner.

MATERIALS AND METHODS

cDNA and chromosomal localization

In our previous work we isolated P1-specific cDNA clones with a combined open reading frame of about 400 codons lacking the 5′ coding sequences (Hu et al., 1993). To isolate the remaining sections of the P1 coding region we used a 5′ fragment of the known P1 cDNA as a probe to screen two commercial cDNA libraries, the Unizap HeLa cell cDNA library of Stratagene and the HeLa S3 5′-stretch library of Clontech. The sequences of the new clones were determined by the dideoxy chain termination method (Sanger et al., 1977) and submitted to the EMBL data bank (accession number: X62153/4).

Human chromosomes were prepared for in situ chromosome hybridization from colcemid-treated lymphocytes according to standard techniques. The procedures for pretreatment of slides, hybridization with 3H-labelled cDNA and staining of human chromosomes have been described (Kunze et al., 1989).

Cell culture conditions

Human HeLa S3 cells (ATCC CCL 2.2) and HeLa 229 cells (ATCC CCL 2.1) were cultivated on 145 mm dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum. For synchronization, subconfluent HeLa S3 cell cultures were treated for 14 hours with 40 ng/ml nocodazole (Pagano et al., 1992). Mitotic cells were shaken off, washed three times with phosphate-buffered saline (PBS), twice with DMEM (10% fetal calf serum) and replated in DMEM plus 10% fetal calf serum. We monitored the progression through the cell cycle by two different methods. First, cells on 90 mm dishes were labelled at the indicated times (see Results) for 30 minutes with 3H-thymidine (in 4 ml medium; 2 µCi/ml). The labelled cells were washed three times with cold PBS and scraped off the plates. The cell pellets were resuspended in 1 ml PBS and lysed by addition of sodium dodecyl sulfate (final: 1%). DNA was then precipitated with 10% trichloroacetic acid. Second, cells on 90 mm dishes were trypsinized and fixed in ethanol (final: 50%) and stained with Hoechst 33258 (final concentration: 8 µg/ml). The relative DNA content per cell was then determined by pulse cytofluorometry (ICP-22, Phywe) using about 20,000 cells/sample. The histograms were evaluated graphically according to the method of Dean (1985), with modifications (G. Adam, personal communication).

Antibodies

P1 cDNA was expressed in bacteria using the pRSET expression system as suggested by the supplier (Invitrogen). The bacterially expressed proteins were used to raise antibodies in rabbits as described (Hu et al., 1993). Monospecific antibodies were isolated using the following affinity purification procedure: the antigen was coupled to Epoxy-activated Sepharose as recommended by the supplier (Pharmacia); antiserum was added and washed extensively, first with Tris-buffered saline (TBS) and then with TBS plus 0.02% Tween, followed by TBS plus 0.02% Tween and 0.5 M NaCl. Bound antibodies were eluted with 0.2 M NaCl in 0.2 M glycine-HCl, pH 2, and immediately neutralized by addition of 1 M Tris-HCl, pH 8.8. The specificity of the P1 antibodies was demonstrated in the following ways. In western blots, performed with total HeLa cell protein extracts, the antibodies only react with the P1 protein and do not cross-react with other cellular proteins, including any one of the six or seven nuclear proteins belonging to the P1-related protein family (Hu et al., 1993). We have also performed immunoprecipitation experiments (see below) and detected, after extensive washing with detergent-containing buffers, only the P1 protein in the precipitates.

RNA and protein blotting

About 2×10⁶ synchronized HeLa cells were washed with PBS and treated according to the method of Chirgwin et al. (1979) for RNA extraction. Total RNA was separated on polyacrylamide gels and blotted on Hybond N membranes (Amersham) essentially as described by Thomas (1980). Endogenous rRNA served as size marker and was visualized by staining with methylene blue. The filters were hybridized with radioactively labelled P1-specific cDNA according to standard procedures (Sambrook et al., 1989). Control hybridizations with histone H4 cDNA and with DNA, complementary to 28 S RNA, were performed as described (Thömmes et al., 1992).

For protein blotting (western blotting) we used 2.5×10⁶ synchronized HeLa cells, which were first washed with PBS and then lysed in RIPA buffer composed of 150 mM NaCl, 1% Nonidet 40 (NP40), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 8. After sonification, samples of the extract, containing identical amounts of protein, were separated on denaturing polyacrylamide gels (Laemmli, 1970) and blotted onto Teflon membranes for staining with P1-specific antibodies essentially according to Towbin et al. (1979).

In vivo phosphorylation

HeLa cells were synchronized as described above. At 3 hours before the indicated times (see below, Fig. 4), medium was removed, and the cells were washed three times with phosphate-free medium (Gibco), before incubation in phosphate-free medium, containing 500 µCi [32P]orthophosphate for 3 hours at 35°C with gentle agitation. The cells were washed in wash buffer (100 mM NaCl, 15 mM sodium citrate, 50 mM sodium fluoride, 0.1 mM sodium vanadate), scraped off the plate and centrifuged briefly (200 g). The cell pellet was resuspended and lysed in 1 ml extraction buffer (450 mM NaCl, 50 mM sodium fluoride, 0.1 mM sodium vanadate, 10 mM sodium bisulfite, 10 mM mercaptoethanol, 10% glycerol, 0.5% Triton X-100, 30 mM Tris-HCl, pH 8). After 40 minutes on ice, insoluble material was removed by centrifugation at 100,000 g. The supernatant was desalted on PD10 columns (Pharmacia) and transferred to extraction buffer with 100 mM NaCl.

The P1 protein was analyzed by immunoprecipitation. Supernatants, corresponding to about 5×10⁶ cells, were incubated with 7.5 µg affinity-purified P1 antibodies for 2 hours on ice. Sufficient amounts of Protein A, coupled to Sepharose (Pharmacia), were added and incubation was continued for 45 minutes with gentle agitation. The pellets were washed in wash buffer (100 mM NaCl, 50 mM sodium fluoride, 0.1 mM sodium vanadate, 5 mM EDTA, 0.1% NP 40, 50 mM Tris-HCl, pH8), NET B (NET A, but 500 mM NaCl) and NET C (NET A, but 150 mM NaCl). The pellets were finally resuspended in loading buffer and investigated by denaturing gel electrophoresis (Laemmli, 1970). Proteins were transferred onto a Teflon membrane, and incorporated radioactivity was measured by autoradiography followed by laser densitometry. The same Teflon membrane was then stained with P1-specific antibodies (Towbin et al., 1979) to determine the amount of immunoprecipitated P1 protein.

Indirect immunofluorescence

HeLa cells were cultivated on coverslips and fixed with 3.5% paraformaldehyde in TBS (Tris-buffered saline: 170 mM NaCl; 10 mM Tris-HCl, pH 7.5). Permeabilization was achieved by 8 minutes with 0.2% Triton X-100 in TBS. After blocking for 15 minutes with 3% bovine serum albumin in TBS, monospecific P1 anti-
bodies (in TBS with 3% bovine serum albumin) were added and incubation was continued for 2 hours at room temperature. As second antibody we used mouse anti-rabbit antibodies, coupled to rhodamine (Boehringer Mannheim), for 30 minutes at room temperature. Nuclear DNA was stained with DAPI (4′,6-diamidino-2-phenylindole-dihydrochloride; 0.04 ng/ml). An inverted Zeiss IM microscope was used as described (Schlatterer et al., 1992) with the filter sets for rhodamine (BP 546; FT 580; LP 590) or DAPI (G 365; FT 395; LP 435). Photographs were taken with a Kodak Ektachrome film (400 ASA).

RESULTS

Amino acid sequence of human protein P1
The combined overlapping P1-specific cDNA sequences comprise a total of 3,050 nucleotides with a 3′ non-translated section of 572 nucleotides ending in a poly(A) stretch, located 16 nucleotides downstream of the polyadenylation signal AATAAA.

The translational start was assumed to be the first in-frame methionine codon. This codon is part of a perfect Kozak consensus sequence of translational start sites (Kozak, 1991). The known cDNA sequence extends for 30 additional nucleotides upstream of the presumed translation initiation codon, but primer extension experiments (Boorstein and Craig, 1989) indicate that the total size of the 5′ non-translated region is 51 nucleotides (not shown). Experimental evidence for the proposed translational start site was obtained by linking the combined cDNAs to the phage T7 promoter for the in vitro synthesis of mRNA. In vitro translation of this mRNA in a reticulocyte extract resulted in a polypeptide migrating in the Laemmli gel electrophoresis system (8% polyacrylamide) exactly in the same position as the P1 protein from HeLa cell nuclei (not shown).

The open reading frame between the first methionine codon and the stop codon could encode a polypeptide of 808 amino acids. Comparison with the yeast Mcm 3 protein reveals extended regions of identical amino acid sequences (Fig. 1A), but also shows that the human protein is significantly shorter than its yeast homologue, which is composed of 971 amino acids (Yan et al., 1991). The yeast Mcm 3 protein has longer N-terminal and C-terminal extensions and longer stretches of amino acids separating the highly conserved homology regions I, II and III (Fig. 1B). Homology region II is composed of smaller units or boxes of conserved amino acids which are common to all known P1-related yeast or mammalian proteins (indicated in Fig. 1A; Hu et al., 1993). These common units were analyzed by Koonin (1993) and found to be distantly related to nucleotide binding sites which include the sequence DEFD (Fig. 1B), a variation of the wide-spread DEAD motif of putative helicases (see: Hu et al., 1993).

The human P1 protein like the yeast Mcm 3 protein contains a high percentage of hydrophilic amino acids (16% arginine and lysine; 16% glutamic acid and aspartic acid) of which many are clustered in a C-terminal domain (P1 protein residues: 655–715; Fig. 1C). A hydrophilic C-terminal domain, although not located within a homology region, is also a feature of the yeast Mcm 3 protein as indicated in Fig. 1B. The high local concentrations of charged amino acids may explain why the P1 protein with a calculated molecular mass of 91 kDa migrates as a 105 kDa polypeptide in denaturing gel electrophoresis (Matagne et al., 1991). A similar discrepancy between the actual and the electrophoretically determined molecular mass has been described for the yeast Mcm 3 protein (Yan et al., 1991).

Chromosomal localization
We have used the P1-specific cDNA to determine the chromosomal localization of the human P1 gene by in situ chromosome hybridization. A significant number of autoradiographic grains were detected on the proximal short arm of chromosome 6 (Fig. 2), indicating that the chromosomal locus for human P1 is 6p12. This result implies that there is probably only one P1 gene in the human genome.

A few other gene loci have previously been mapped to the proximal short arm of human chromosome 6 including genes GST2, encoding glutathione S-transferase, and KRAS1P, a pseudogene of human K-ras oncogene (Trent and Ziegler, 1993). But, in general, it is a relatively uncharted region of the human genome, and the mapped human P1 gene may be a useful marker for its further exploration.

Expression in synchronized cell cultures
For synchronization, HeLa S3 cell cultures were uniformly blocked in G2 phase/mitosis with the microtubule inhibitor nocodazole. Accumulated mitotic cells were shaken off and replated in fresh medium. Most replated cells entered the cell cycle and started to replicate their DNA synchronously at 10–12 hours after release from the mitotic block as demonstrated by pulse-labelling with [3H]thymidine (Fig. 3A).

We have prepared protein extracts at various times after release from nocodazole inhibition and determined the amount of P1 protein by western blotting. We detected no significant variation in the amounts of P1 protein during progression into S phase (Fig. 3B). In fact, evidence suggests that P1 may be a rather stable protein because the level of P1 did not detectably decrease in HeLa cells treated for 5 hours with cycloheximide to inhibit protein synthesis. However, after 16 hours in cycloheximide the amount of P1 was found to be only 20-30% of the control (determined by western blotting, not shown). A low rate of turnover of P1 protein is also suggested by the observation that immunoprecipitated P1 contained very little incorporated radioactivity after cultivating HeLa cells for 4 hours in the presence of [35S]methionine under conditions known to effectively label DNA polymerase α and other nuclear proteins (Thömmes et al., 1986) (data not shown).

In previous experiments with primary human embryo lung fibroblasts, synchronized by serum starvation, a several-fold increase in P1-specific mRNA levels was observed at the G1–to-S phase transition (Thömmes et al., 1992). To determine whether a similar increase also occurs in nocodazole-synchronized cells, total RNA was extracted at various times after removal of nocodazole and used for electrophoresis and RNA blotting. Hybridization with 32P-labelled P1 cDNA revealed that P1-specific mRNA levels were relatively low during the first 8 hours, but increased by factors of 5-10 during S phase (Fig. 3C). The increase in P1-specific mRNA coincided with that of histone H4 mRNA whose synthesis is known to be coupled to the S phase (Osley, 1991) (Fig. 3B). The amounts of 28 S rRNA in the RNA samples tested were not significantly different, showing that similar amounts of total RNA were added to the gel lanes (Fig. 3C). The results shown in Fig. 3 imply that the additional mRNA synthesized during S phase is
Fig. 1. Deduced amino acid sequence of the human protein P1 in comparison with its yeast homologue, the Mcm 3 protein. (A) Aligned sequences. Amino acids at identical positions are boxed. Large boxes define regions of high sequence homology. Sequence motifs, highly conserved between nine yeast and mammalian Mcm/P1-related proteins, are underlined (Hu et al., 1993). (B) Location of homology regions I, II and III in human protein P1 and yeast protein Mcm3. The position of the DEFD motif, the signature sequence of all known Mcm/P1 proteins (Koonin, 1993), is indicated. The open boxes indicate the positions of the hydrophilic C-terminal domains. Amino acid residues are numbered. (C) Hydrophilicity plot of human protein P1. Note the block of hydrophilic amino acids between residues 660 and 750.
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not, or not immediately, used for translation and P1 protein synthesis. Thus, if P1 acts at the beginning of, or during, S phase as suggested by a comparison with the homologous yeast protein, it must be regulated by means other than de novo synthesis. We have tested one possibility, cell-cycle-dependent phosphorylation.

**Protein phosphorylation**

We labelled synchronized HeLa cells at different times after removal of nocodazole with $[^{32}P]$ phosphate (see Materials and Methods). In this experiment, we determined the relative DNA content of cells by impulse cytofluorometry, which allows an unambiguous evaluation of the fraction of cells in different phases of the cell cycle (Fig. 4A). Protein extracts were prepared from synchronized cells and treated with P1-specific antibodies for immunoprecipitation under experimental conditions known to effectively and specifically precipitate more than 90% of P1 protein from crude extracts. Autoradiography and laser densitometry showed a 3- to 4-fold increase in the rate of P1 phosphorylation at the beginning of S phase (Fig. 4B; 10 hours and 14 hours). The rate of P1 phosphorylation decreased at the end of S phase (18 hours) and in G2 (24 hours). In a control experiment, synchronized cells in late S phase were arrested again by nocodazole and then released to initiate a second cell cycle. At 10 hours after removal of nocodazole, the fraction of cells in S phase had increased (Fig. 4A), just like the amount of $[^{32}P]$ phosphate in immunoprecipitated protein P1 (Fig. 4B, N + 10).

Kimura et al. (1994) have recently performed similar experiments with the mouse P1 protein in 3T3 cells, synchronized by serum starvation. They also observed an S-phase-dependent increase in the rate of P1 phosphorylation, which, however, continued to rise until mitosis (Fig. 7, of Kimura et al., 1994). The reason for the discrepancy between their data and the results shown in Fig. 4 is not known, but could be due to the different cell types analyzed.

In any case, our results are consistent with the notion that phosphorylation may affect the function of P1 during S phase, but we need further investigation to confirm this hypothesis.
have no information concerning the protein kinase involved in P1 phosphorylation in vivo. We note though that cAMP-dependent protein kinase and p34\(^{cd2}\) kinase effectively use bacterially expressed P1 as a substrate for phosphorylation in vitro (unpublished). A potential phosphorylation site for the cAMP-dependent kinase (Kemp and Pearson, 1990) was found in the homology region I (P1 protein residues 157-161: RRYSD; Fig. 1A); and variations of the cdc2-kinase consensus (S/T-P-X-K/R) (Nigg, 1993) are found in homology regions I (P1 residues 112-115: SPPT), II (P1 residues 465-468: TPME) and III (residues 611-613: SPVT; Fig. 1A). But future experiments should show whether any one of these sites is used for phosphorylation in vivo.

Computer search also reveals that the P1 protein contains numerous and widely distributed potential phosphorylation sites for casein kinase (S/T-X-E/D) (Kemp and Pearson, 1990), and our preliminary results show that P1 is exclusively phosphorylated at threonine and serine residues (unpublished).

**Nuclear localization**

An important result from studies with yeast cells was that the proteins Mcm 2, Mcm 3 and Mcm 5 enter the nucleus at the end of mitosis, remain in the nucleus during most of G\(_1\) phase.

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**Fig. 4.** Cell-cycle-dependent phosphorylation of P1. (A) Relative DNA contents. HeLa cells were synchronized by release from nocodazole inhibition. At the indicated times, the cells were stained with Hoechst 33258 and the fluorescence intensity (representing the DNA content) in about 2\(\times\)10\(^4\) cells was determined by impulse cytofluorometry. The data were quantified as described by Dean (1985) with modifications (G. Adam, personal communication). We show the percentage of cells in different cell cycle phases. (B) Cells were labelled with \([^{32}P]\)phosphate after removal of nocodazole at the times indicated. N + 10, synchronized cells in late S phase (18 hours) were blocked for 8 hours with nocodazole, mitotic cells were harvested, released for a second cell cycle and labeled with \([^{32}P]\)phosphate as described (Materials and Methods) before preparing the extracts 10 hours after removal of the drug. Extracted proteins were immunoprecipitated, separated by polyacrylamide gel electrophoresis, transferred to a Teflon membrane and analyzed by autoradiography. Densitometry revealed that amounts of incorporated label increased by factors of about 3 and 4 at 10 hours and 14 hours, respectively, relative to the 8 hour value. The N+10 value is 2.1 times higher than the control at 8 hours. (C) The Teflon membrane, used for autoradiography in (B) was stained with P1 specific antibodies to compare the amounts of proteins in the gel.

**Fig. 5.** Intracellular distribution of protein P1. (A) HeLa S3 cells in phase contrast microscopy; (B) DAPI staining of nuclear DNA; (C) immunofluorescence with affinity-purified P1-specific antibodies and secondary rhodamine-coupled anti-rabbit antibodies. Bar, 20 \(\mu\)m.
Expression of nuclear protein P1 and disappear at the beginning of S phase. This behaviour was interpreted to indicate that Mcm proteins function early during S phase and are then excluded, as one mechanism of preventing the initiation of additional replication cycles in one S phase (Hennessy et al., 1990; Yan et al., 1993).

We were interested in determining whether the nuclear localization of P1 protein, a mammalian counterpart of the yeast Mcm 3 protein, was also regulated in a cell-cycle-dependent manner. For that purpose, we performed indirect immunofluorescence assays with affinity-purified P1 antibodies to determine the localization of P1 in synchronized HeLa S3 cells, and found, in agreement with previous studies (Thömmes et al., 1992), a localization of P1 in the nucleus. Significantly, the protein remained in the nucleus at all times after release from the mitotic block (not shown).

We have performed additional experiments with HeLa 229 cells, which have a flatter cell morphology and a higher cytoplasm/nucleus ratio, and therefore allow a clearer demonstration of nuclear staining. A disadvantage is that HeLa 229 cells cannot as easily be synchronized as HeLa S3 cells by the nocodazole procedure because mitotic cells remain more tightly bound to the substratum and cannot reproducibly be shaken off for replating.

We have applied the immunofluorescence procedure to actively proliferating HeLa 229 cells under subconfluent culture conditions and screened many hundred immunostained cells. We expected that in these cultures about one third of the cells would be in S phase (length of S phase/total cell cycle length: 6-8 hours/20-24 hours). However, we could not detect any interphase cell with a significantly reduced nuclear and a more intense cytoplasmic staining, in agreement with the results obtained for synchronized HeLa S3 cells. In Fig. 5 we show an example of immuno-stained HeLa 229 cells demonstrating the predominant nuclear localization of immunostainable P1 protein. A faint staining of the cytoplasm was detected in all cells, but not in control experiments using the second antibody only (not shown). It is therefore quite likely that a minor fraction of P1 occurs in the cytoplasm of proliferating HeLa cells.

The sample, shown in Fig. 5, includes a cell in mitosis with a rounded shape (A) and a denser DAPI staining of DNA (B) Immuno-staining reveals a distribution of P1 protein over the entire mitotic cell (C). The dispersal of stainable P1 protein was observed in all mitotic cells examined.

The left-hand panels (A) of Fig. 6 show three rounded cells in metaphase while the right-hand panels (B) show a cell in late telophase. The dispersal of P1 throughout the mitotic cells is quite apparent in all cases. In addition, the chromosomal regions of mitotic cells appear darker in immunofluorescence and therefore contain less bound P1 antibodies than the remaining parts of the cell. This result suggests that P1 may be excluded from chromosomes in mitosis.

The results just described are in agreement with recent studies on the P1 protein in mouse 3T3 cells (Kimura et al., 1994) and the human BM28 protein, a homologue of the yeast Mcm 2 protein (Todorov et al., 1994). Immunofluorescence experiments with BM28-specific antibodies show an exclusive nuclear localization of the BM28 protein also, during most of the cell cycle, and its dispersal throughout the entire cell in mitosis.

DISCUSSION

The human nuclear protein P1 belongs to an interesting novel
class of mammalian proteins which may play a role in the initiation of genome replication. We have investigated its expression and nuclear localization in HeLa cells progressing from mitotic arrest through G1 phase into the S phase of the cell cycle.

The human genome most likely contains a single P1-encoding gene which is activated at the beginning of the S phase resulting in a 5- to 10-fold increase in P1-specific mRNA levels in HeLa cells. But in spite of this, the total amounts of P1 protein do not detectably change when HeLa cells proceed through G1 phase into S phase, and experimental evidence indicates that the P1 protein is rather stable and has a low turnover rate. In addition, HeLa cells contain well over 10^5 P1 molecules/nucleus as estimated by comparing western blots of total extractable proteins with western blots of known amounts of purified bacterially expressed P1 (unpublished). Thus, it is unlikely that the function of protein P1 is regulated by changes in the number of protein molecules.

Amino acid sequence homologies suggest that protein P1 like its yeast counterpart, the Mcm3 protein, may be involved in the regulation of genome replication. In fact, Yan et al. (1993) proposed that the yeast Mcm 3 protein may be related to the hypothetical licensing factor because protein Mcm3 (and other related nuclear yeast proteins) disappear from the nucleus during S phase. The licensing factor is thought to act at the beginning of S phase and later to be degraded, inactivated or excluded from the nucleus. New factor for the next cycle will gain access to chromatin sites only after a breakdown of the nuclear envelope during mitosis (Blow and Laskey, 1988). However, in contrast to the homologous yeast protein, human P1 remains in the nucleus during and after S phase. But in mitosis, protein P1 is dispersed throughout the cell and appears to be largely excluded from chromosomes. It is enclosed in nuclei again upon formation of new nuclear membranes after mitotic telophase. Thus, protein P1 must be regulated during S phase by mechanisms different from those operating in yeast cells. One mechanism could be protein phosphorylation, since the rate of P1 phosphorylation increases severalfold at the beginning of S phase. Thus, if P1 protein acts as a licensing factor at the beginning of S phase, its later inactivation may be connected with a phosphorylation reaction.

While this paper was under revision, a publication by Kimura et al. (1994) appeared describing experiments on the P1 protein in mouse cells. The authors showed that a soluble P1 fraction in 3T3 cell nuclei is hyperphosphorylated during S phase and also concluded that phosphorylation may be required for inactivation of P1 after it has performed an initiation function in early S phase. This is certainly an interesting possibility, but the effects of phosphorylation can only be assessed when a biochemical function for P1 or any one of the related yeast or mammalian proteins has been identified.

In conclusion, the high degree of sequence conservation between the yeast and the mammalian nuclear proteins suggests related functions, but the present experiments show that these functions may be regulated differently in the two organisms. A major mechanism of regulation in yeast cells may be the S-phase-dependent exclusion of Mcm proteins from nuclei, whereas other mechanisms must be active in mammalian cells. A central event could be a cell-cycle-dependent differential phosphorylation of P1 proteins.

We thank Gerold Adam for discussions and for his help with the evaluation of cytophotometric data. This work was supported by grants from the Deutsche Forschungsgemeinschaft (through SFB 156) and the German-Israeli Fund for Research and Development. D. S. is a recipient of a Friedrich-Naumann-Fellowship, and R. B. is supported by Boehringer-Ingelheim-Fonds.

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(Received 4 July 1994, Accepted 6 December 1994).