A human nuclear protein with sequence homology to a family of early S phase proteins is required for entry into S phase and for cell division

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SUMMARY

Molecular cloning and characterisation of a human nuclear protein designated BM28 is reported. On the amino acid level this 892 amino acid protein, migrating on SDS-gels as a 125 kDa polypeptide, shares areas of significant similarity with a recently defined family of early S phase proteins. The members of this family, the *Saccharomyces cerevisiae* Mcm2p, Mcm3p, Cdc46p/Mcm5p, the *Schizosaccharomyces pombe* Cdc21p and the mouse protein P1 are considered to be involved in the onset of DNA replication. The highest similarity was found with Mcm2p (42% identity over the whole length and higher than 75% over a conservative region of 215 amino acid residues), suggesting that BM28 could represent the human homologue of the *S. cerevisiae* MCM2.

Using antibodies raised against the recombinant BM28 the corresponding antigen was found to be localised in the nuclei of various mammalian cells. Microinjection of anti-BM28 antibody into synchronised mouse NIH3T3 or human HeLa cells presents evidence for the involvement of the protein in cell cycle progression. When injected in G1 phase the anti-BM28 antibody inhibits the onset of subsequent DNA synthesis as tested by the incorporation of bromodeoxyuridine. Microinjection during the S phase had no effect on DNA synthesis, but inhibits cell division. The data suggest that the nuclear protein BM28 is required for two events of the cell cycle, for the onset of DNA replication and for cell division.

Key words: cell division, early S phase protein family, entry into S phase, MCM2, nuclear protein BM28

INTRODUCTION

In the eukaryotic cell cycle there are two crucial transition steps - the onset of replication of the chromosomal DNA and the entry into cell division. These two steps, separated by the cell cycle phase termed G2, seem to be coupled (Enoch and Nurse, 1991). A possible idea is that the same proteins could take part in the regulation and/or the execution of both events. An example is the complex of the protein kinase cdc2 with cyclins, which has been demonstrated to play an important role in the cell cycle of yeast and of mammalian cells (Draetta, 1990; Nurse, 1990). Cyclin A has been reported to be required in two points of the mammalian cell cycle, in the entry into S phase (Girard et al., 1991; Pagano et al., 1992) and in the G2/M transition (Pagano et al., 1992), suggesting its possible role in events regulating the dependence of mitosis on completion of DNA replication (Walker and Maller, 1991).

Recently a new family of putative replication proteins (Yan et al., 1991; Hennessy et al., 1991; Chen et al., 1992) and *Schizosaccharomyces pombe* Cdc21p (Coxon et al., 1992), but also in mouse cells (the protein P1: Thommes et al., 1992). MCM2 and MCM3 (Gibson et al., 1990; Yan et al., 1991) are essential genes identified by mutations that affect the mitotic stability of minichromosomes. CDC46 was identified as a suppressor of the mutations of two other S-phase-related *cdc* genes, *CDC45* and *CDC54* (Moir and Botstein, 1982), and was found to be identical with the *MCM5* gene (Chen et al., 1992). It has been shown to be involved in the early steps of DNA replication (Hennessy et al., 1990), acting in a narrow window before the hydroxyurea-sensitive elongation step (Hennessy et al., 1991; Chen et al., 1992). Cdc21p of *S. pombe* was identified by selecting cdc mutants affecting S phase and nuclear division and may act before the elongation step of DNA replication (Coxon et al., 1992). The mouse nuclear protein P1 was identified as a part of the multiprotein ‘holoenzyme’ of the replication-specific DNA polymerase α-primase. It displays a strong homology with the *S. cerevisiae* Mcm3p and it was reported that it represents the mammalian homologue of the yeast protein (Thommes et al., 1992). Therefore, the proteins of this family are believed to...
be related to the onset of DNA replication. In the timing of the yeast cell cycle the actions of these genes appear to be interposed between the G1 activation of the Cdc28p/Cdc2p kinase and the initiation of DNA replication (Li and Alberts, 1992). Their possible relation with the initiation of DNA replication is still unclear. However, at least for Mcm2p, Mcm3p and Mcm5p/Cdc46p, their function appears to have a direct bearing on the efficiency of replication origin function (Yan et al., 1991; Chen et al., 1992). These findings place the members of the newly defined family of early S phase proteins close to the proteins of the origin recognition complex (ORC) (Bell and Stillman, 1992; Diffley and Cocker, 1992).

Here we report the molecular cloning, sequencing and characterisation of a human cDNA, encoding a protein, designated (according to the clone number of its cDNA) as BM28, which shares areas of significant homology with members of the newly defined family of early S phase genes/proteins mentioned above. By microinjections of a polyclonal antibody raised against the recombinant protein we present evidence that BM28 is required for two cell cycle events, the onset of DNA replication and cell division.

MATERIALS AND METHODS

cDNA library screening
cDNAs containing the largest inserts were obtained using the size-selected (inserts larger than 2.2 kb) CaCoII Agt11 cDNA library derived from human colon carcinoma cells, kindly provided by Dr Ned Mantei, ETH, Zurich (Franke et al., 1989). Recombinant phages were plated with Escherichia coli Y1088 cells and blotted onto Hybond-N membranes (132 mm diameter, 30000 plaques/plate). Replicas were screened with the 1.1 kb EcoRI/SphI insert of the clone 6A9 described earlier (Todorov et al., 1991), radiolabelled with [α-32P]dCTP using the oligolabelling kit of Pharmacia. Hybridisation was carried out in 0.25 M sodium phosphate, pH 7.2, 7% sodium dodecyl sulphate (SDS) at 65°C (Church and Gilbert, 1984). The was carried out in 0.25 M sodium phosphate, pH 7.2, 7% sodium dodecyl sulphate (SDS) at 65°C (Church and Gilbert, 1984). The hybridisation buffer, then 

nucleotide sequence was determined by the cycle sequencing, and cell division.

Expression and purification of the recombinant protein (BM28)
The coding sequence of BM28 as well as its two subfragments (5’ end, 1.8 kb Ncol/NcoI fragment, pep2; and 3’ end, 1.6 kb NcoI/EcoRI fragment, pep3) were recloned in the pRESET vector designed to express recombinant N- and C-terminal polypeptides. (N, Ncol; P, PstI; E, EcoRI; S, SphI). Thick bars indicate the coding region of BM28.

Production of antibodies and immunochemistry
Rabbits were injected with 0.5 mg of purified recombinant pep2 (N-terminal part of the BM28 molecule) or pep3 (C-terminal part of the molecule) emulsified in complete Freund’s adjuvant. Subsequently, the rabbits received three injections with the same amount of protein in incomplete Freund’s adjuvant. For affinity purification of the antibodies 5 mg of each protein (pep2 or pep3) were covalently coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia). The sera were diluted with 1 volume of PBS containing 0.1% Triton X-100 and passed at least five times through the respective column. The Sepharose was then washed with 20 volumes of PBS/Triton X-100 and eluted with 0.1 M glycine-HCl, 0.15 M NaCl, pH 2.8. The fractions were neutralised immediately with 0.1 volume of 1 M Tris-HCl, pH 8.0, and dialysed against PBS. Prior to their use in control injections, solutions containing purified rabbit IgGs were also dialysed for 24 hours against PBS.

For immunoblotting, total cellular proteins were separated on 10% polyacrylamide/SDS gels (50 μg cell lysate per track) and electroblotted to nitrocellulose (Schleicher & Schuell, BA85). After blocking, the blots were incubated for 16 hours in a suitable dilution of the antibodies, and after washing and a second blocking, in a solution of 125I-labelled anti-rabbit immunoglobulins (Amersham).

Immunostaining of cells for mitotin was performed as described previously (Todorov et al., 1992). For staining BM28 methanol- and acetone-fixed cells were incubated with the first antibody diluted in PBS up to 1:100 for 1 hour at room temperature. After several washes the coverslips were incubated with FITC- or Texas Red-conjugated anti-rabbit IgG (Dianova, Hamburg) for another hour. Moni-
toring of bromodeoxyuridine incorporation was performed as described (Pepperkok et al., 1991; Pagano et al., 1992).

For double staining with anti-BrdU and anti-BM28 antibodies methanol-fixed cells were digested by endonuclease/exonuclease procedures essentially as described by Fox et al. (1991) to create single-stranded regions in DNA and then labelled with the antibodies.

**Cell culture, synchronisation of cells and microinjection protocol**

HeLa cells were obtained from the cell culture facility of the German Cancer Research Centre and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplied with 10% foetal calf serum and antibiotics. Potoroo (rat kangaroo) kidney PtK2 cells (ATCC CCL56) were obtained from the American Type Culture Collection. They were grown in DMEM supplemented with 10% foetal calf serum (FCS), 1 mM glutamine and antibiotics.

IMR90 and NIH3T3 cells were cultured as previously described (Pepperkok et al., 1988; Sorrentino et al., 1990). For synchronisation in G0 phase they were seeded on glass coverslips (10 mm × 10 mm) and after reaching 50% confluency they were starved for 3 days in culture medium containing 0.5% FCS. After this period 90% of the cells were in G0 phase as determined by FACS analysis and failure to incorporate BrdU within a labelling period of 12 hours. G0-synchronised cells could be activated to re-enter the cell cycle by addition of 20% FCS. Under these conditions they synchronously started to incorporate BrdU (entry into S phase) between 12 and 16 hours after activation.

HeLa cells were synchronised by treatment with nocodazole (50 ng/ml) for 10 hours as described (Morla et al., 1989; Pagano et al., 1992). Then, cultures were washed once with PBS and mitotic cells were carefully shaken off and subsequently replated onto glass coverslips prepared for microinjection. Until 10 hours after replating more than 90% of the cells had a DNA content corresponding to the G1 phase of the cell cycle as analysed by flow cytometry. Thereafter, cells started to enter S phase as detected by labelling with BrdU (see Fig. 9).

To synchronise NIH3T3 cells at the early S phase, G0-synchronised cells were growth activated for 24 hours with culture medium containing 20% FCS in the presence of hydroxyurea (HU, 3 mM final concentration) which arrests cells at the beginning of S phase. Under these conditions, more than 95% of the cells were blocked in early S phase as determined by FACS analysis and failure to incorporate BrdU. They were released from the HU block by washing them 3 times in PBS for 5 minutes and further incubation in HU-free culture medium. At 6 hours after the release, on average more than 90% of the cells had incorporated BrdU.

HeLa cells synchronised by mitotic shake off were arrested in early S phase by a similar procedure by addition of 3 mM HU to the culture medium immediately after replating.

Microinjection experiments were performed with an automatic system (AIS, Zeiss) as described (Ansorge and Pepperkok, 1988).

**Immunoprecipitation**

For immunoprecipitation extracts from HeLa or NIH3T3 cells metabolically labelled with a mixture of 35S-containing compounds highly enriched in methionine (Trans-35S-label, ICN-Flow). For this labelling cells were grown to 50-70% confluency, washed and incubated in methionine-free MEM (Gibco), containing 10% dialysed foetal calf serum and 0.2 mM/ml Trans-35S-label for 4 hours. All operations afterwards were performed at 4°C. The cells were washed and scraped with PBS, containing 1 mM PMSF and 20 mM methionine and lysed by sonication in 10 volumes immunoprecipitation buffer (150 mM NaCl, 2 mM EDTA, 20 mM NaF, 25 mM Tris-HCl, pH 7.5, 20 mM methionine, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF, 20 µg/ml aprotinin, 20 µg/ml leupeptin). The lysate was clarified by centrifugation at 14,000 g for 15 minutes and preabsorbed with 100 µl/ml Protein G-Sepharose (Pharmacia) and 50 µl/ml pre-immune rabbit serum for 1 hour. The Sepharose was spun down and the preabsorbed lysate was immunoprecipitated with 40 µg/ml affinity-purified anti-BM28 (anti-pep2) immunoglobulins and 50 µl/ml Protein G-Sepharose for 4 hours. The beads were washed three times in immunoprecipitation buffer, followed by threefold washing in immunoprecipitation buffer containing 1 M NaCl (Laliberte et al., 1984), once more in immunoprecipitation buffer and finally with PBS containing 1 mM PMSF. The immunoprecipitated proteins were extracted by boiling in electrophoresis sample buffer and separated on SDS-polyacrylamide gels.

**Image analysis**

Quantification of BrdU-specific nuclear fluorescence was performed as described (Pepperkok et al., 1991, 1993). Briefly, fluorescence was observed with an inverted microscope (Zeiss) and images of stained cells were digitised using a SIT low light level camera coupled to a DVS image enhancement system (both from Hamamatsu). Off-line analysis of digitised images was performed with an IBAS image analysis system (Zeiss). The average fluorescence intensity of single nuclei (Iav) and the area (A) that they occupied in the image were determined and the integrated optical intensity (IOD), which is proportional to the amount of incorporated BrdU, was calculated by the formula: IOD = Iav × A.

**RESULTS**

**Molecular cloning and identification of BM28**

The cloning of BM28 was achieved with the help of a partial 1.1 kb cDNA clone (designated 6A9, Fig. 1) isolated previously (Todorov et al., 1991). In a hybrid selected translation assay, this 6A9 clone resulted in a poly peptide product with a molecular mass of 125 kDa, which could be immunoprecipitated with the antibody against the proliferation-associated nuclear protein mitotin (Philippova et al., 1987). In northern blot hybridisation the 6A9 probe recognises a single poly(A)+ RNA of 4.5 kb (Todorov et al., 1991). The 6A9 clone was used for hybridisation screening of several λgt10 and λgt11 human RNA-derived cDNA libraries. The largest cDNAs were obtained with a size-selected human colon carcinoma cell RNA-derived CDNA library (see Franke et al., 1989), enriched in CDNA inserts larger than 2 kb. In screening this library, several clones larger than 3 kb, which hybridised with the 6A9 probe under stringent conditions, were identified. The largest CDNA insert (3.4 kb), designated BM28, was cloned, sequenced as described in Materials and Methods and further analysed.

**Sequence analysis**

The complete sequence of the of BM28 clone (3376 bp), (EMBL Data Library, accession number X67334) contains an open reading frame of 892 codons, capable of coding for a polypeptide of 99 kDa. Database searches revealed that the BM28 cDNA and its product share stringents of significant similarity (Fig. 2A) with five genes/proteins that are considered to be related to the early steps of DNA replication: the S. cerevisiae MCM2p, MCM3p (Gibson et al., 1990; Yan et al., 1991) and Cdc46p/Mcm5p (Chen et al., 1992); the S. pombe Cdc21p (Coxon et al., 1992) and the mouse protein P1 (Thommes et al., 1992).

The sequence of the human BM28 shows the closest relation with the S. cerevisiae MCM2 protein. The similarity between the BM28 and the MCM2 protein is rather high over the full
Fig. 2. Comparison of the complete BM28 amino acid sequence with related proteins. (A) The BM28 protein sequence (on top) is compared with Mcm2p, Mcm3p, the mouse protein P1, Cdc21p and Cdc46p/Mcm5p. The alignment was generated using the CLUSTALV program (Higgins and Sharp, 1989). Identical residues in different sequences are boxed. Identical residues in BM28 and MCM2 are marked with asterisks. (B) Alignment of BM28-related protein sequences (uppercase names) with part of the transcription activation domain of a family of prokaryotic transcription factors related to nifA and ntrC (lowercase names). CLUSTALV was used to align separate alignments of the two protein families (fixed gap penalty, 12; floating gap penalty, 10, PAM250 amino acid weight matrix). The output was produced using Prettyplot (GGC), using a threshold parameter of 1.0 and a plurality parameter of 9 to allow identical and chemically similar amino acids to be boxed. The accession numbers of the sequences shown are: BM28, X67334; MCM2, P29469; CDC46, P29496; MCM3, P24279; P1, P25206; CDC21, P29458; YBR1441, Z21487; algB, P23737; dcdD, P113632; flaA, P19323; flaB, P17899; nifA, S06965; tyrR, P07604; xylR, P06519; ntrC, P03029. (C) Putative zinc binding motif identified in the sequences of BM28 and MCM2.
length of the amino acid sequence, reaching an overall level of identity of 42%. In addition there are several areas of higher similarity: BM28 amino acid positions 260-431, 465-677, 703-758. The longest region (amino acid positions 465-677), has a length of more than 200 amino acids with a level of identity over 75%. The level of identity with the other four proteins in this region is as high as 50-60%.

BM28 also shows weak similarity with a family of prokaryotic transcription factors, related to nifA, as recently pointed out by Koonin (1993) for the MCM proteins. A profile alignment of the BM28-related proteins, and the set of nifA-related transcription factors (Kustu et al., 1991) is shown in Fig. 2B. The conserved sequences include a putative ATP/GTP-binding site in the prokaryotic proteins, although the eukaryotic proteins have GXx[Al/S]xK instead of the GXxGK motif A consensus (Walkcr et al., 1982). Koonin has suggested that this may represent a modified A consensus, and it will be of interest to determine whether the BM28 protein shows nucleotide-binding or NTPase activity.

BM28 contains a putative metal-binding motif of the form CX3CX19CX2C (Fig. 2C) that is conserved in the closely related Mcm2p sequence (Yan et al., 1991), but this complete motif is not found in the other related proteins. This sequence does not correspond to consensus sequences for the DNA-binding `zinc fingers’ found in transcription factors (see Coleman, 1992, for a review), although similar motifs have been shown to be involved in zinc-binding in a variety of proteins, including for instance eukaryotic RNA polymerases (Treich et al., 1991).

Characterisation and cellular localisation of the BM28 product

The BM28 cDNA was recloned into a pRSET vector and expressed in E. coli. The resulting protein migrated on SDS-gels as a 125 kDa polypeptide (Fig. 3), which is larger than the expected molecular mass of 99 kDa derived from the cDNA sequence. This discrepancy could be explained by an amino acid sequence feature causing an irregular rate of migration on SDS-gels. The recombinant protein showed the same migration characteristics as the in vitro translation product of in vitro transcripts from the BM28 cDNA in the pBluescript vector (data not shown). We also expressed two sub-fragments of the BM28 cDNA: (a) a 1.8 kb Ncol/Ncol fragment starting at the first ATG; and (b) a 1.6 kb Ncol/EcoRI fragment containing.
the next 300 codons to the 3′ end of the coding sequence (Fig. 1). The corresponding protein products were designated pep2 and pep3, respectively. pep2 migrates on SDS-gels as a polypeptide of 90 kDa, which is also higher compared to the molecular mass of 70 kDa predicted by the cDNA sequence. pep3 migrates as expected at a position of a 38 kDa polypeptide. These data indicate that the N-terminal section of the BM28 protein contains sequences responsible for its irregular rate of migration, not proportional to the molecular mass. A similar discrepancy between the predicted and apparent molecular sizes has been reported for the MCM3 protein (Gibson et al., 1990), sharing significant similarities with BM28 (see Fig. 2).

Purified pep2 and pep3 were used to raise antibodies in rabbits. The antibodies were purified on affinity columns containing the corresponding polypeptides. These antibodies reacted on western blots with the complete BM28 protein as a polypeptide of 90 kDa, which is also higher compared to the molecular mass of 70 kDa predicted by the cDNA sequence. pep3 migrates as expected at a position of a 38 kDa polypeptide. These data indicate that the N-terminal section of the BM28 protein contains sequences responsible for its irregular rate of migration, not proportional to the molecular mass. A similar discrepancy between the predicted and apparent molecular sizes has been reported for the MCM3 protein (Gibson et al., 1990), sharing significant similarities with BM28 (see Fig. 2).

Affinity-purified antibodies against pep2 and pep3 were used to localise the BM28 in fixed cells by immunofluorescence. In parallel experiments both antibodies gave always the same pattern of staining. In all interphase cells we observed a nuclear distribution of BM28 that was similar for different cell lines: HeLa (Fig. 5A), PtK2 (Fig. 6) and 3T3 cells (Fig. 5E). In PtK2 cells, which are flatter and allow the study of the fine morphology, we could detect BM28 also in centrosomes (Fig. 6A-C) and their derivatives - the poles of the mitotic spindle (Fig. 6G,H). The centrosomal association of the BM28 antigen could also be seen in HeLa cells, but the staining intensity was weaker compared to PtK2 cells (Fig. 5A). During mitosis BM28 becomes redistributed in the extrachromosomal region in late prophase (Fig. 5C), metaphase and in anaphase cells (Figs 5B, 6G,H). In telophase the entire antigen is found in the newly forming nuclei (Figs 5D, 6I). A clear co-localisation of BM28 with BrdU staining was not detected even after very short pulses with BrdU (Fig. 5E,F).

Comparing the BM28 protein with the nuclear antigen mitotin, which was identified by the monoclonal antibody used to clone BM28 (Todorov et al., 1991), we found several similarities: mobility in SDS-gels, pl, high levels in proliferating cells as compared to resting cells (Philipova et al., 1991, and unpublished data) and nearly constant rates of expression during the cell cycle (Zhelev et al., 1990; Todorov et al., 1991). These data suggested that we had cloned the cDNA encoding the protein mitotin (Todorov et al., 1991). However, the immunofluorescence data presented here, obtained with the affinity-purified polyclonal antibodies against the two different regions of the BM28 protein, are not completely consistent with this suggestion. As shown in Fig. 6, BM28 and mitotin show different subnuclear distribution. Further we could not find an accumulation of BM28 during the G2/M phase of the cell cycle, as it was reported for mitotin (Todorov et al., 1988; see also Fig. 6). In addition the nuclear antigen mitotin was found to be resistant to various extraction procedures being presumably part of the ‘nuclear matrix’ structures (Philipova et al., 1987), while the BM28 is easily extractable from the cell (results not shown). It is thus likely that BM28 and mitotin are two distinct proteins, perhaps showing sequence similarities or having shared epitopes.

**Nuclear microinjection of anti-BM28 antibody inhibits the onset of S phase**

The high level of sequence similarity between BM28 and a family of proteins with functions related to the onset of S phase...
suggested that BM28 might play a similar role. To test this hypothesis the affinity-purified antibody prepared against pep2, the N-terminal polypeptide of BM28 (anti-BM28), was microinjected into the nucleus of cells synchronised in G1 phase. Cell cycle progression was analysed by their capacity to incorporate BrdU, indicating that they had entered S phase.

HeLa cells were synchronised by nocodazole treatment and mitotic cells were released from this block by shaking off and replating them in fresh medium (for details see Materials and Methods). They were microinjected 4 hours after replating and DNA synthesis was monitored by pulse labelling them 20 hours after replating for 2 hours with BrdU. IMR90 and NIH3T3 cells were arrested in G0 phase by serum starvation (see Materials and Methods). Six hours prior to microinjection they were growth activated with 20% foetal calf serum. Immediately after injection the cells were labelled with BrdU, fixed and immunostained 24 hours after growth activation.

For all three cell types tested, microinjection of the anti-BM28 antibody resulted in a marked decrease in the number of BrdU-positive cells compared to noninjected cells (Fig. 7, Table 1), suggesting that BM28 is required for cell cycle progression. This growth inhibitory effect could be significantly reversed by co-injection of anti-BM28 and an eukaryotic expression construct (pXBM28) containing the complete BM28 coding sequence under the control of the CMV promoter. Microinjection of pXBM28 alone resulted in an increased expression of BM28 in the nucleus, which was already detectable by immunofluorescence staining for BM28 at 6 hours after microinjection.

**Table 1. Effect of anti-BM28 antibody microinjection on DNA synthesis**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Material injected*</th>
<th>%BrdU-positive cells†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Injected</td>
<td>Not injected</td>
</tr>
<tr>
<td>HeLa</td>
<td>Anti-BM28</td>
<td>31±5</td>
</tr>
<tr>
<td></td>
<td>Anti-BM28 + pXBM28‡</td>
<td>52±2</td>
</tr>
<tr>
<td></td>
<td>Rabbit IgG</td>
<td>73±6</td>
</tr>
<tr>
<td>IMR90</td>
<td>Anti-BM28</td>
<td>10±7</td>
</tr>
<tr>
<td></td>
<td>Anti-BM28 + pXBM28‡</td>
<td>52±2</td>
</tr>
<tr>
<td></td>
<td>Rabbit IgG</td>
<td>54±6</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Anti-BM28</td>
<td>23±3</td>
</tr>
<tr>
<td></td>
<td>Anti-BM28 + pXBM28‡</td>
<td>66±8</td>
</tr>
</tbody>
</table>

*Experimental conditions are described in the legend to Fig. 7.
†The average values and standard deviations of about 250 injected cells were analysed.
‡The eukaryotic expression vector pXBM28 contains the complete BM28 cDNA under the control of the CMV promoter. It was injected in a final concentration of 0.1 mg/ml together with the anti-BM28 antibody (2 mg/ml).

Fig. 5. Localisation of BM28 antigen in mouse NIH3T3 and human HeLa cells. Immunofluorescence microscopy of HeLa cells (A,B) and NIH3T3 cells (D-F) in different phases of the cell cycle (interphase, A,E; prophase, C; metaphase and anaphase, B; telophase, D). The cells were stained with the anti-BM28 (anti-pep2) antibody (A-D) and a Texas Red-conjugated anti-rabbit IgG. The cells in (E-F) are double-stained with the anti-BM28 antibody/Texas Red-conjugated anti-rabbit IgG (E) and an FITC-conjugated anti-BrdU antibody (F). Prior to fixation cells had been pulse-labelled with BrdU for 30 minutes. The same immunolocalisation results were obtained using anti-pep3 antibody (not shown) The positions of centrosomes are indicated by arrows. Bar, 10 μm.
Fig. 6. Parallel localisation of BM28 antigen and mitotin. PtK₂ cells are double-stained with the anti-pep2 (anti-BM28) antibody (A-C,G-I) and the anti-mitotin monoclonal antibody (D-F,K-M). Cells in interphase and mitotic sub-phases could be distinguished: interphase and prophase cells (A-F); metaphase (G,K); anaphase (H,L); and telophase (I,M). Arrowheads indicate the positions of centrosomes and spindle poles. Bar, 10 µm.
A putative human homologue of yeast Mcm2p

(data not shown). No significant effect on DNA synthesis, compared to noninjected cells, was detected when cells were microinjected with purified control rabbit IgGs.

To characterise the growth-inhibitory effect caused by microinjection of the anti-BM28 antibody two types of experiments were performed. First, G1-synchronised HeLa cells were microinjected with anti-BM28 6 hours after release from the nocodazole block. They were pulse-labelled with BrdU for 2 hours at different time-points thereafter and the number of cells incorporating BrdU was determined. Up to 12 hours after replating, the number of BrdU-positive cells was below 10% for both anti-BM28 and control IgG-injected cells (not shown), indicating that at these time-points the majority of the cells were still in G1 phase. At time-points later than 12 hours after replating the number of BrdU-positive cells increased with time for anti-BM28 and control IgG-injected cells (Fig. 8). However, the number of BrdU-positive cells injected with anti-BM28 increased more slowly compared to those cells that received control IgG. We conclude that the microinjection of the anti-BM28 does not cause a block, but rather a delay in the entry into S phase. The subsequent recovery of cells from the inhibition in cell growth could be explained by a degradation of injected antibody.

Second, HeLa or NIH3T3 cells were microinjected at different time-points after replating or growth activation with 20% FCS, respectively. For both cell lines a significant inhibition of DNA synthesis was observed when anti-BM28 was microinjected before cells had entered S phase (Fig. 9A, 4 and 8 hours; Fig. 9B, 6-16 hours). In contrast to this, little or no
Effect on DNA synthesis was observed for those time-points where the majority of the cells had already entered S phase prior to microinjection (Fig. 9A, 12-16 hours; Fig. 9B, 20 hours). In the NIH3T3 cells the inhibitory effect was more pronounced when microinjections were performed in G1 phase (4 hours after the release from the block); late G1 phase (8 hours); and at various stages in S phase (12-16 hours). At 16 hours after the release they were pulse-labelled with BrdU for 2 hours, fixed and immunostained as described in legend to Fig. 7. Under these conditions, cells started to enter S phase (detected by BrdU incorporation) between 12 and 16 hours after growth activation with FCS and had a maximum of S phase cells at 24 hours. Only those microinjections of anti-BM28 that had been performed before cells had entered S phase caused significant inhibition of DNA synthesis.

Then they were microinjected with anti-BM28 and 30 minutes later the cells were transferred into fresh medium without HU. BrdU incorporation was monitored at different time-points after release from the HU block (Fig. 10). For all time-points tested, cells microinjected with anti-BM28 showed a BrdU-specific nuclear fluorescence comparable to that obtained for noninjected cells (Fig. 10). This finding was statistically confirmed by quantitative image analysis of the BrdU-specific nuclear fluorescence intensities in at least 100 injected and noninjected cells and we concluded that the anti-BM28 antibody does not inhibit progression through S phase.

### Table 2. Effect of anti-BM28 antibody microinjection on cell division

<table>
<thead>
<tr>
<th>Time of injection (hours after release from HU block)</th>
<th>Number of positively stained cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-BM28+ anti-BM28 antibody+pXBM28 Rabbit IgG Rabbit IgG+pXBM28</td>
<td></td>
</tr>
<tr>
<td>HeLa cells</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>97±11</td>
</tr>
<tr>
<td>10</td>
<td>103±18</td>
</tr>
<tr>
<td>NIH3T3 cells</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>98±13</td>
</tr>
<tr>
<td>10</td>
<td>115±11</td>
</tr>
</tbody>
</table>

HeLa and NIH3T3 cells were synchronised in early S phase by a hydroxyurea block as described in Materials and Methods. Immediately before or 10 hours after release from the HU block anti-BM28 antibody, anti-BM28 antibody+pXBM28 or control rabbit IgG were injected into the nuclei of exactly 100 single separated cells. At 24 hours after release from the block cells were fixed and stained with Texas Red-conjugated anti-rabbit IgG and the number of positively stained cells was counted. The means of two independent experiments are shown, n.d., not determined.

### Nuclear microinjection of anti-BM28 antibody inhibits cell division

In further experiments we tested the effect of the anti-BM28 antibody on cell division. HeLa and NIH3T3 cells were synchronised in early S phase by treatment with HU. Then exactly 100 cells were microinjected into the nucleus each with the anti-BM28 antibody or control IgG, and the HU block was released by addition of fresh medium. At 24 hours later the cells were fixed, stained with Texas Red-conjugated anti-rabbit IgG and the number of positively stained cells was counted. When control IgG was injected, the number of the positively stained cells was almost twice as high as the number (100) of cells injected initially, suggesting that most of the cells divided within 24 hours (Table 2). This was supported by the finding that the majority of the cells displayed a cytoplasmic and nuclear distribution of the injected antibody, suggesting that it had been redistributed during mitosis (not shown). In contrast to this, less then 15% of the cells injected with the anti-BM28 antibody had divided during the same period, and the majority of the cells retained the nuclear localisation of the injected antibody. Similar results were obtained when cells were microinjected 10 hours after the release of the HU block (Table 2), where the majority of cells were in late S or in G2/M phase as determined by FACS analysis and BrdU incorporation (not shown). The inhibitory effect on cell division was significantly reversed by co-injection with the pXBM28 construct, allowing expression of BM28. Such reversal was seen only in the case of early S phase injection (immediately after the HU block). In late injections (10 hours
A putative human homologue of yeast Mcm2p

... poor or no reversion was detected. This could be explained by the lack of time for expression of the recombinant protein up to mitosis.

In no experiment could an accumulation of M phase cells be observed in anti-BM28-injected cells, thus excluding the hypothesis that anti-BM28 antibodies block cells during mitosis. The results suggest that BM28 is required for cell cycle progression between S phase and mitosis.

**DISCUSSION**

This paper presents the results on the molecular cloning of a human cDNA and characterisation of its product (BM28), which belongs to a family of putative replication proteins, first identified in yeast (Yan et al., 1991; Hennessy et al., 1991; Coxon et al., 1992). The BM28 protein is most closely related to \textit{S. cerevisiae} Mcm2p, raising the question of whether BM28 represents the human homologue of the yeast protein and we are currently testing whether yeast \textit{mcm2} mutants can be complemented by the BM28 gene. Consistent with published data on Cdc46p (Hennessy et al., 1990), BM28 shows a predominantly nuclear localisation, and the distribution of the antigen in the nuclei of all interphase cells is similar to the picture reported for the mouse P1 protein (Thommes et al., 1992). We observed rather poor colocalisation between BM28 and the sites of DNA replication in mammalian cells visualised by incorporation of BrdU (see Fig. 6), indicating that BM28 is not concentrated in ‘replication factories’ (Hozak et al., 1993) but has a much more widespread distribution in the nucleus. We have not detected a dramatic cell-cycle-dependent redistribution of BM28 as shown for Cdc46p in \textit{S. cerevisiae} cells (Hennessy et al., 1991).

Our results on the inhibition of the onset of DNA synthesis in various mammalian cells by microinjection of anti-BM28 antibody in G1 phase are consistent with a role for BM28 in DNA replication. Analogous results have been obtained with the yeast \textit{cdc46} mutant, which arrests with a single genome equivalent of DNA (Hennessy, et al., 1991). Yeast mutants defective in the \textit{mcm2}, \textit{mcm3} and \textit{cdc21} genes are able to replicate most of their DNA at the restrictive conditions (Gibson et al., 1990; Yan et al., 1991; Coxon et al., 1992), but...
this may reflect ‘leakiness’ of the available conditional alleles. The failure to arrest ongoing DNA replication with anti-BM28 antibody suggests a role for this protein in initiation rather than elongation, but this result is a little difficult to interpret as it could be argued that BM28 assembled into functioning replication complexes might be inaccessible to antibody. In any case these data do not exclude the possibility of an involvement of BM28 in the formation of the origin recognition complex (see Bell and Stillman, 1992). Further experiments in support of this hypothesis could employ the anti-BM28 antibody(ies) to isolate mammalian (human) ORCs by immunoprecipitation.

Inhibition of cell division is observed when anti-BM28-antibody is injected in early S phase or later, while it does not apparently interfere with the progression of DNA synthesis. The effect of the anti-BM28 antibody microinjection on cell division could thus be independent of its effect on the onset of DNA synthesis and BM28 could be another protein, like cyclin A (Pagano et al., 1992), that is required at more than one point of the mammalian cell cycle. The localisation of BM28 antigen within human cells suggests that it is present in two cellular compartments, the cell nucleus and the centrosomes, and this might be related to the distinct activities of DNA replication and cell division that are inhibited by microinjection of antibody. Cytoplasmic microinjection experiments are required to confirm any involvement of centrosomal BM28 in the events leading the cell to division.

An alternative interpretation that we cannot exclude is that the anti-BM28 antibody subtly interferes with an ongoing S phase, perhaps causing chromosome damage, incomplete DNA replication, or interferes with some other aspect of chromosome function, and this damage is sensed by a ‘checkpoint’ system that blocks mitosis and cell division (reviewed by Hartwell and Weinert, 1989). This question could be most easily addressed in yeast, assuming Mcm2p does represent the BM28 homologue. If the S. cerevisiae Mcm2p is required for both DNA replication and entry into mitosis, mcm2 mutant cells should still arrest in G2 when combined with checkpoint mutations that allow mitosis if replication is incomplete or DNA is damaged. Alternatively, if Mcm2p is only required for S phase, checkpoint mutations should allow mcm2 mutant cells to proceed through mitosis and cell division before terminal arrest. It would also be of interest to determine the phenotype of related mutants, when combined with ‘checkpoint’ mutations.

The data on the cellular localisation suggest that BM28 and mitotin could be different, although closely related, proteins. It is possible that the proliferating cell nuclear antigen mitotin, identified by means of a monoclonal antibody, could represent an antigenically related protein or proteins, sharing epitope(s) with BM28. Thus mitotin may be another human homologue of the yeast protein family. Another possibility is that the antimitotin monoclonal antibody recognises only one or few modified forms of BM28. It is very likely that human cells contain a number of BM28-related proteins, since there are at least five members of this family in S. cerevisiae (see Coxon et al., 1992; Bussereau et al., 1993) and it is possible that the complexity of gene families is greater in multicellular organisms.

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