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Negative Regulation of DNA Replication by the Retinoblastoma Protein Is Mediated by Its Association with MCM7

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A yeast two-hybrid screen was employed to identify human proteins that specifically bind the amino-terminal 400 amino acids of the retinoblastoma (Rb) protein. Two independent cDNAs resulting from this screen were found to encode the carboxy-terminal 137 amino acids of MCM7, a member of a family of proteins that comprise replication licensing factor. Full-length Rb and MCM7 form protein complexes in vitro, and the amino termini of two Rb-related proteins, p107 and p130, also bind MCM7. Protein complexes between Rb and MCM7 were also detected in anti-Rb immunoprecipitates prepared from human cells. The amino-termini of Rb and p130 strongly inhibited DNA replication in an MCM7-dependent fashion in a *Xenopus* in vitro DNA replication assay system. These data provide the first evidence that Rb and Rb-related proteins can directly regulate DNA replication and that components of licensing factor are targets of the products of tumor suppressor genes.

The retinoblastoma (Rb) susceptibility gene, *Rb-1*, is paradigmatic for a class of evolutionarily conserved genes variously termed tumor suppressor genes, anti-oncogenes, or recessive oncogenes. Deletion or mutational inactivation of *Rb-1* is associated with the genesis of a variety of human cancers, including retinoblastoma, osteosarcoma, and small cell lung, bladder, and breast carcinomas (for a review, see reference 83). Mice hemizygous for Rb function are predisposed to a distinct spectrum of neoplasms, exhibiting an increased susceptibility to the development of brain, pituitary, and thyroid tumors (11, 43, 49). In addition to negatively regulating cell proliferation, Rb also functions to induce and/or maintain cell differentiation. For example, mice nullizygous for Rb function perish in utero and exhibit defects in the differentiation of hematopoietic, nervous, lens, and muscle tissues (11, 43, 49, 56, 61, 73, 88). Rb is also involved in a distinct pathway of deregulated cell growth and tumorigenesis, which is transformation induced by DNA tumor viruses. The E1A protein of adenovirus, large-T antigens of simian virus 40 (SV40) and polyomaviruses, and the E7 protein of human papillomaviruses all form physical complexes with the Rb protein, and such interactions abrogate Rb-mediated growth suppression (16, 22, 23, 63, 85). That each of these virus families has evolved independently to bind Rb underscores the idea that this tumor suppressor gene functions at one or more critical checkpoints in the regulation of cell growth.

Rb-1 encodes a ubiquitously expressed set of nuclear proteins, termed p105-Rb, that are subject to cyclical waves of phosphorylation by cyclin-dependent kinases (7, 8, 51, 55, 60, 80, 83). Quiescent, terminally differentiated cells and cells in early portions of the cell cycle carry largely unphosphorylated p105-Rb. Shortly before the initiation of DNA synthesis, Rb is phosphorylated by cyclin D- and E-associated kinases and becomes increasingly modified as cells progress through S phase and G_2 (15, 83). Rb is abruptly dephosphorylated at the end of mitosis probably by a type I protein phosphatase activated in anaphase (54, 55, 65). Since Rb phosphorylation appears to be a prerequisite for transit through the G_1/S boundary, it is widely suspected that phosphorylation inactivates at least one growth-suppressing function of Rb. This supposition is buoyed by the observations that (i) certain viral oncoproteins preferentially bind to unphosphorylated p105-Rb and (ii) Rb alleles carrying mutations at various sites of phosphorylation show increased potency as negative regulators of cell cycle progression (31, 53).

Rb is a member of a family of genes that includes p107 and p130 (25, 32, 52, 59, 89). Although mutations of p107 or p130 have not yet been associated with human neoplasia, evidence from nullizygous animals indicates that these Rb-related proteins play an important supportive role in the regulation of cell proliferation and differentiation (12, 50). In contrast to Rb-negative animals, mice nullizygous for p107 or p130 function are not predisposed to tumorigenesis and do not show obvious physical or behavioral abnormalities. Nonetheless, it is clear that the functions of Rb, p107, and p130 at least partially overlap, since mice nullizygous for p130 and p107 exhibit developmental abnormalities, such as neonatal lethality, defective bone development, and shortened limbs, and $Rb^{+/-}$/$p107^{-/-}$ animals show increased mortality, dysplastic retinal lesions, and growth retardation. Overlapping functions of Rb family members have also been noted in vitro. For example, deregulated skeletal muscle cell differentiation in $Rb^{-/-}$ cells can be corrected by the enforced expression of exogenous p107 (73). Yet this overlap is not universal, since p107 has been shown to suppress the growth of C-33A human cervical carcinoma cells, an Rb-negative tumor line, whereas Rb does not (89). Consistent with observations suggesting that certain functions of Rb family members may be redundant, Rb, p107, and

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p130 show significant structural similarities, and each of these proteins interact with a similar set of viral and cellular proteins (25, 32, 52, 59, 89).

Molecular analyses of various human tumors indicate that the carboxy-terminal two-thirds of *Rb-1* is a frequent target of mutation (36–38). Such mutations are often quite subtle, leading to amino acid substitutions or splicing defects that eliminate specific carboxy-terminal exons from the Rb protein. These data imply that the integrity of this mutational hotspot, which is often referred to as the Rb pocket, is required for Rb-mediated growth suppression. Consistent with this idea, microinjection of the Rb pocket into human tumor cells arrests cell cycle progression and has suggested that Rb's growth-limiting function is restricted to a discrete temporal window approximately 6 h prior to the initiation of S phase (29, 30). Microinjection of the Rb pocket into synchronized cells subsequent to this window of Rb sensitivity had little or no effect on DNA synthesis. These results suggest that the Rb pocket is critically important for the regulation of transit through a G_1 checkpoint, subsequent to which a cell is committed to replicating DNA. In accord with this suggestion, this portion of p105-Rb is also bound by viral oncoproteins and is the site of interaction of Rb with a bevy of cellular targets and regulators, such as transcription factors (e.g., E2F-1) and cyclin-cyclin-dependent kinase (cdk) kinases (37).

Three observations have suggested that the amino terminus of the Rb protein is also likely to play an important role in growth suppression. First, the Rb amino terminus is well-conserved across a variety of mammalian species, and portions are closely related to the amino termini of p107 and p130 (25, 32, 52, 59, 89). Second, mutations within the Rb amino terminus have been detected in retinoblastoma tumors, and such mutations leave intact the vast majority of the Rb protein, including the Rb pocket (18, 35). Thus, lesions within the Rb amino terminus also result in loss-of-function mutations. Finally, a variety of mutations engineered within the Rb amino terminus block (i) Rb phosphorylation, (ii) in vitro Rb-mediated growth suppression, and (iii) differentiation and tumor suppression in vivo despite the retention of wild-type levels of E2F-binding activity within the Rb pocket (69, 70, 84). These observations suggest that the Rb amino terminus may be a site for interaction with targets or regulators of Rb function. Indeed, akin to analyses of the Rb pocket, the amino terminus of Rb has been shown to bind a number of cellular proteins, including p84, a nuclear matrix protein, $TAF_{II}250$, hsc73, and RbK, a cell-cycle regulated kinase (20, 42, 75, 78, 79). Additionally, evidence from partial proteolysis and electron microscopy has suggested that at least two structural domains are encoded by the Rb amino terminus and that these domains facilitate oligomerization (33). Interestingly, although the amino termini of Rb, p107, and p130 share significant regions of amino acid identity, several functional distinctions have been noted. For example, RbK preferentially associates with the Rb amino terminus, and transcription factor Sp1 binds the amino terminus of p107 (14, 79).

To understand further the function(s) of the Rb amino terminus, we employed a yeast two-hybrid screen to isolate human cDNAs encoding proteins that specifically associate with this portion of Rb. Here, we report that one of the cDNAs that we have isolated encodes MCM7, a recently identified member of a family of proteins involved in the initiation of DNA replication. We provide evidence that (i) the amino termini of Rb, p107, and p130 bind MCM7 in vitro, (ii) Rb may be found in association with MCM7 in vivo, and (iii) the physical interaction of Rb and p130 with MCM7 inhibits DNA replication in vitro.

MATERIALS AND METHODS

Yeast two-hybrid screen. A human cDNA fragment encoding the amino-terminal 380 amino acids of Rb was subcloned into pAS2 (creating 5′RbpAS2), a yeast multicopy plasmid carrying the GAL4 DNA-binding domain (a kind gift of Steven J. Elledge, Baylor University, Houston, Tex. [19]). A yeast strain, Y190 (*his3 leu2 trp1*), which carries GAL4-dependent *lacZ* and *HIS3* genes, was transformed with 5′RbpAS2, and TRP^+ transformants were examined for *lacZ* and *HIS3* expression via a colorimetric assay and growth in medium lacking histidine. Residual growth of TRP^+ transformants on His^- plates was eliminated by the inclusion of 25 mM 3-aminotriazole. A $lacZ^-$ $HIS3^-$ $TRP1^+$ cell clone was subsequently transformed with a HeLa cDNA library (Matchmaker system; Clontech Laboratories, Inc.) fused to the GAL4-activation domain in plasmid pGAD-GH, a *LEU2*-containing multicopy yeast plasmid, and 6×10^5 LEU^+ transformants were replica plated onto indicator plates to score for histidine prototrophy and in situ β-galactosidase activity. This selection and screen resulted in the isolation of 19 candidate $lacZ^+$ HIS^+ colonies that were examined further to determine whether their phenotype required the presence of 5′RbpAS2. Each of the 19 colonies was grown in rich medium, and LEU^+ cells that had lost 5′RbpAS2 were selected via growth on His^+ Trp^+ Leu^- plates supplemented with cyclohexamide. Replica plating of resulting colonies indicated that descendants of each of the 19 candidate colonies were histidine auxotrophs and devoid of β-galactosidase activity. Further analyses showed that the HeLa cDNAs carried by each of the 19 candidate colonies interacted specifically with GAL4-Rb and not other bait constructs such as GAL4 fusions with *p53*, c-*myc*, *lamin*, *SNF1*, and *DP-1*.

A 600-bp partial MCM7 cDNA (designated MCM7c) isolated in this screen was employed as a hybridization probe to screen a λZAPII HeLa library (Stratagene, Inc., La Jolla, Calif.) according to standard procedures. Double-stranded dideoxy sequencing was performed with Sequenase 2.0 (U.S. Biochemicals, Inc., Cleveland, Ohio) to determine the sequence of the longest MCM7 cDNA (designated MCM7n) obtained in this screen.

In vitro protein-binding assays. MCM7n and MCM7c were expressed as glutathione *S*-transferase (GST) fusion proteins via the insertion of their respective cDNAs at the *Eco*RI site of pGEX2TK (Pharmacia, Inc., Piscataway, N.J.). GST fusion proteins prepared with the amino termini of human Rb (GST-Rb), human p107 (GST-p107), human p130 (GST-130), and a *Schistosoma* surface antigen (GST-FSH15) have previously been described (78, 79). Expression of GST fusion proteins was induced in BL21 bacteria by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside, and fusion proteins were harvested and quantified as previously described (78, 79). For in vitro translation reactions, MCM7n and MCM7c cDNAs were subcloned at the *Eco*RI site of pTM1 (62). pTM1 constructions carrying wild-type and mutated human Rb cDNAs were gifts of Dennis J. Templeton (Case Western Reserve University, Cleveland, Ohio). In vitro translation reactions were prepared by using a proprietary rabbit reticulocyte kit (TnT-coupled transcription-translation system; Promega, Inc., Madison, Wis.) according to instructions provided by the manufacturer. For in vitro protein-binding assays, in vitro translation reaction mixtures were diluted to 1:50 in EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of pepstatin A and leupeptin per ml) and incubated with GST fusion proteins bound to glutathione-agarose beads (Sigma, Inc., St. Louis, Mo.) for 60 min at 4°C. Following incubation, bead-bound proteins were eluted by boiling in 2% sodium dodecyl sulfate (SDS) and resolved by electrophoresis through acrylamide gels.

***Xenopus* extract preparation and DNA replication assays.** Interphase *Xenopus* egg extracts and demembranated sperm nuclei were prepared and utilized as described elsewhere (77). For replication reactions, cytosolic and membrane fractions were thawed, reconstituted, and supplemented with a system for the regeneration of ATP (20 mM phosphocreatine, 50-μg/ml creatine kinase, 2 mM ATP). Demembranated sperm chromatin was added to egg extracts (500 per μl of extract), and replication of sperm chromatin was monitored at various time points by agarose gel electrophoresis following incubation for 20 min with [α-^{32}P]dCTP. For replication assays that included GST fusion proteins, each fusion protein was added to egg extracts to a final concentration of 3.3 ng/μl, and the mixture was incubated on ice for 30 min prior to the addition of sperm nuclei. To demonstrate dependence on Rb-MCM7 interactions, a maltose-binding protein (MBP)-MCM7c fusion protein was prepared by subcloning a MCM7c cDNA fragment at the *Eco*RI site of pMALc-2 (New England Biolabs, Inc., Beverly, Mass.). MBP and MBP-MCM7c fusion proteins were prepared according to the instructions of the manufacturer (New England Biolabs, Inc.). Equal quantities of MBP or MBP-MCM7c fusion protein were mixed with GST fusion proteins and incubated on ice for 30 min prior to their addition to replication assays.

Nuclear assembly, nuclear transport, and H1 kinase assays. Nuclear assembly was assessed according to a previously described protocol (77). Nuclear transport was assayed with rhodamine-labeled human serum albumin coupled to multiple copies of the SV40 large-T antigen nuclear localization signal (a kind gift of Douglass Forbes, University of California-San Diego, San Diego, Calif. [77]). To determine if GST-Rb bound *Xenopus* cdk2 or otherwise inhibited cdk2 kinase activity, 100 μl of *Xenopus* egg extracts was or was not incubated with GST-Rb, fusion proteins were collected on glutathione-agarose beads, and supernatants were incubated with 20 μl of p13^{suc}-Sepharose beads (equivalent to 100 μg of

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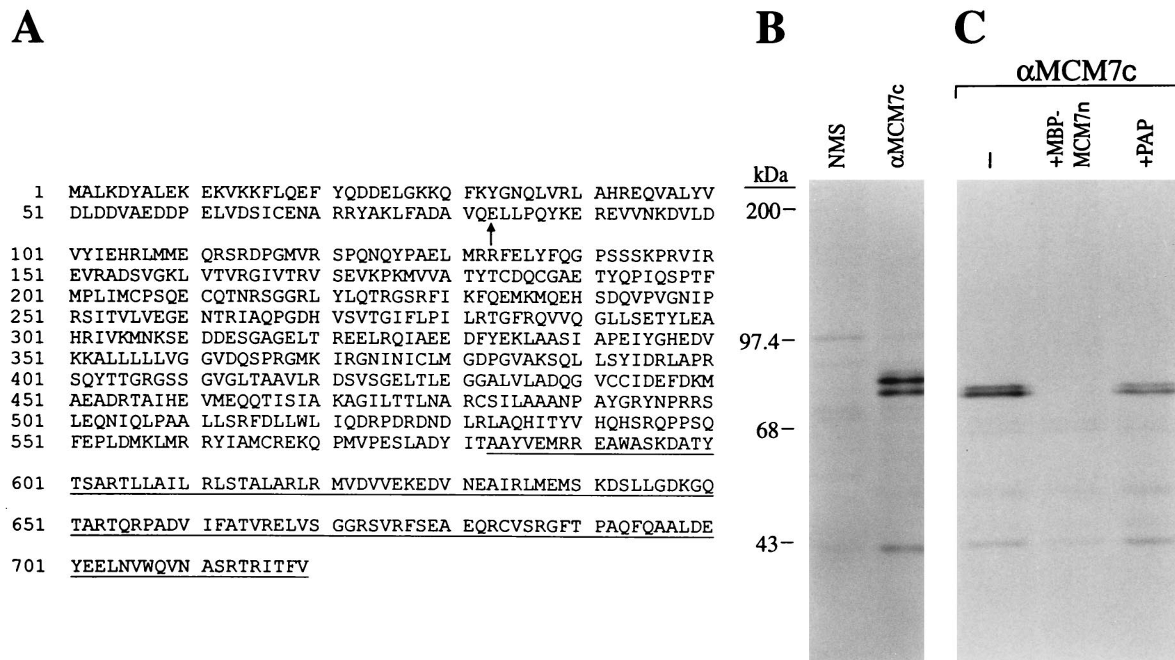

FIG. 1. Sequence of human MCM7 and characterization of MCM7 in human cells. (A) Amino acid sequence of human MCM7 (GenBank accession no. D55716). Underlined amino acids (denoted MCM7c) were encoded by two cDNAs identified in a yeast two-hybrid screen. An arrow indicates the amino-terminal end of the MCM7 protein (denoted MCM7n) encoded by a partial cDNA isolated by hybridization of a human cDNA library. (B) Immunoprecipitation of MCM7 proteins from human cells. ML-1 cells were metabolically labeled with [^{35}S]methionine, nondenatured extracts were incubated with normal mouse serum (NMS) or mouse anti-MCM7c antiserum (αMCM7c), and precipitates were resolved on an 8% polyacrylamide gel. Molecular mass markers are indicated on the left. (C) Characterization of MCM7 proteins precipitated from human cells. As described above, ML-1 extracts were incubated with mouse MCM7c antiserum alone (−) or following preincubation of antiserum with an MBP-MCM7n fusion protein (MBP-MCM7n). The phosphorylation status of MCM7 proteins was assessed via incubation of αMCM7c precipitates with potato acid phosphatase (PAP).

p13*suc* protein) for 30 min at 4°C. Sepharose beads were collected by centrifugation, washed once with 50 mM HEPES (pH 7.2), and resuspended in an equal volume of H1 kinase buffer (50 mM HEPES [pH 7.2], 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μM ATP, 0.1-mg/ml histone H1, 0.5 μCi of [^{32}P]ATP). Following incubation for 20 min at room temperature, samples were boiled in an equal volume of Laemmli sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography at −80°C.

Preparation of human cell extracts, antibodies, and immunoprecipitations. ML-1 cells were cultured in Dulbecco's modified minimal essential medium (GIBCO/BRL, Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, Ga.) and 0.05 mg of mezlin (Miles Laboratories, Inc., West Haven, Conn.) per ml under 5% CO_2 in a humidified incubator at 37°C. ML-1 cells were metabolically labeled as previously described (64). Anti-Rb ascites fluid was prepared from XZ77 hybridoma cells (a gift of Nicholas Dyson, Massachusetts General Hospital Cancer Center, Charlestown, Mass.). To generate polyclonal antisera against human MCM7, New Zealand White rabbits and BALB/c mice were sequentially immunized with GST-MCM7c or GST-MCM7n protein in Freund's complete and incomplete adjuvants. For coimmunoprecipitations, radiolabeled cell extracts were incubated for 60 min on ice with XZ77 ascites fluid, and precipitates were collected on protein A-Sepharose beads. Beads were washed four times in EBC buffer, resuspended in 50 μl of SDS lysis buffer (20 mM Tris [pH 7.5], 50 mM NaCl, 0.5% SDS, 1 mM dithiothreitol), and heated to 95°C for 2 min. Supernatants were removed, diluted with 500 μl of EBC buffer, and incubated with normal mouse serum, anti-MCM7 antiserum, or XZ77 ascites fluid. Precipitates were collected on protein A-Sepharose beads, washed four times with EBC buffer, boiled in Laemmli sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

RESULTS

A yeast two-hybrid screen identifies MCM7 as an amino-terminal Rb-binding protein. To identify human proteins that bind the Rb amino-terminus, a yeast two-hybrid strategy was adopted. To generate 5′RbpAS2, a bait construct for the screening of a human cDNA library, the amino-terminal 380 amino acids of human Rb were fused in frame with the GAL4 DNA-binding domain in pAS2 (19). A yeast strain, Y190, that carries GAL4-dependent *lacZ* and *HIS3* genes, was transformed with 5′RbpAS2, and transformants were examined for their intrinsic ability to drive *lacZ* and *HIS3* expression via a colorimetric assay and growth in medium lacking histidine. A histidine auxotroph lacking β-galactosidase activity was subsequently transformed with a HeLa cDNA library fused to the GAL4 activation domain, and transformants were replica plated onto indicator plates to score for histidine prototrophy and in situ β-galactosidase activity. This selection and screen resulted in the isolation of 19 colonies whose β-galactosidase activity and growth on His$^-$ plates were dependent on the presence of 5′RbpAS2. Further analyses showed that the HeLa cDNAs carried by each of these colonies interacted specifically with GAL4-Rb and not other bait constructs such as GAL4-fusions with *p53*, c-*myc*, *lamin*, *SNF1*, and *DP-1*. Sequence analysis revealed that 15 of 19 candidate cDNAs encoded either short polypeptides or polypeptides translated from previously identified cDNAs in the antisense orientation. However, two colonies, numbers 23 and 24, carried nearly identical partial cDNAs encoding the carboxy-terminal 137 amino acids of MCM7, a member of the minichromosome maintenance (MCM) family of proteins (Fig. 1A [underlined amino acids]) (39, 46). Clones 23 and 24 differed from each other by several amino acids at their respective amino termini that were contributed by the oligonucleotide used to generate the cDNA library. The 137-amino-acid partial-MCM7 protein encoded by cDNAs 23 and 24 was designated MCM7c. cDNA clone 23 was employed subsequently as a hybridization probe to isolate a cDNA encoding the majority of human MCM7 (635 amino

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acids, designated MCM7n) from a HeLa cDNA library (Fig. 1A, arrow).

As a first step toward analyzing Rb-MCM7 interactions, GST fusion proteins encoding MCM7c and MCM7n were purified from bacteria and used as immunogens to generate polyclonal mouse and rabbit anti-MCM7 antibodies. As expected based on previously reported studies of MCM7, rabbit and mouse antisera prepared against both GST-MCM7 fusion proteins precipitated a protein doublet of 82 and 85 kDa from a wide variety of human cell lines (Fig. 1B and data not shown) (28, 74). Immunoprecipitates prepared with control antiserum did not contain these proteins (Fig. 1B [NMS]). As is also shown in Fig. 1C, preincubation of MCM7c antiserum with a bacterially expressed MCM7n fusion protein (MBP-MCM7n) abolished the precipitation of this protein doublet. Two experiments were performed to determine if the 82- and 85-kDa proteins were precipitated via their direct or indirect interaction with anti-MCM7 antisera. First, human cell extracts were examined by Western blotting, and each anti-MCM7 antiserum was shown to react with a broad band of protein of 85 kDa that could not be resolved further (for example, see Fig. 4A). Second, each anti-MCM7 antiserum precipitated both the 82- and 85-kDa proteins from human cell extracts that had been completely denatured by boiling in SDS (data not shown).

MCM7 has been previously noted to encode consensus sites for phosphorylation by a variety of kinases, and we hypothesized that differing extents of phosphorylation might account for the 82- and 85-kDa proteins (28, 74). To address this hypothesis, human cells radiolabeled with [^{32}P]orthophosphate were immunoprecipitated with anti-MCM7 antisera. Such immunoprecipitates did not yield evidence of radiolabeled MCM7 proteins (data not shown). Consistent with this observation, the apparent molecular masses of the 82- and 85-kDa proteins precipitated by MCM7 antiserum were not appreciably altered by incubation of MCM7 precipitates with potato acid phosphatase (Fig. 1C). In contrast, Rb immunoprecipitates were readily dephosphorylated in parallel reactions (data not shown). Thus, it is unlikely that the 82- and 85-kDa forms of MCM7 are distinguished by differing extents of phosphorylation. Additional experiments will be required to determine the features that distinguish the 82- and 85-kDa MCM7 proteins.

MCM7 binds the amino termini of Rb, p130, and p107 in vitro. Although specific physical interactions between the Rb amino terminus and MCM7 were detected in yeast cells, we wished to determine (i) if such interactions might be limited to this cellular milieu and (ii) if MCM7 bound full-length Rb protein. Additionally, we wished to explore the possibility that the amino termini of other Rb family members might also physically interact with MCM7. To address these issues, a series of protein-binding assays were performed with in vitro-translated proteins and GST fusion proteins. In the first experiment, a GST-MCM7n fusion protein was prepared in bacteria, bound to glutathione agarose beads, and incubated with full-length, radiolabeled Rb protein synthesized in rabbit reticulocyte extracts. In a reciprocal experiment, the amino-terminal 380 amino acids of human Rb were expressed as a GST fusion protein and incubated with radiolabeled MCM7n protein synthesized in reticulocyte extracts. As shown in Fig. 2A, Rb bound MCM7n (GST-MCM7n), but not a heterologous GST fusion protein, GST-FSH15. As shown in Fig. 2B (left panel), in vitro translates of MCM7n bound to GST-Rb but not to GST-FSH15. Consistent with results from yeast cells, in vitro-translated MCM7c protein also efficiently bound GST-Rb, indicating that amino acids 583 to 719 of MCM7 are

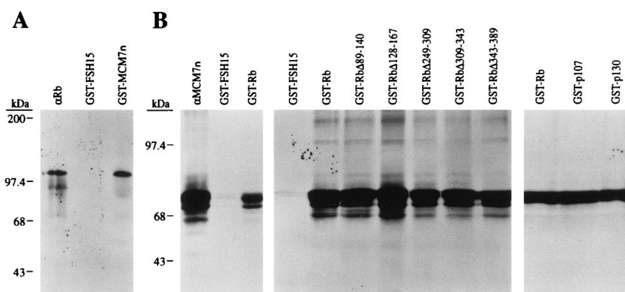

FIG. 2. In vitro protein-binding assays. (A) Protein-binding assays with radiolabeled human Rb protein. Rabbit reticulocyte lysates programmed with human Rb cRNA were incubated with [^{35}S]methionine, and the resulting radiolabeled proteins were divided evenly among three tubes. Aliquots were incubated with an anti-Rb monoclonal antibody (XZ77) or were incubated with equivalent amounts of GST-FSH15 or GST-MCM7n fusion proteins. Immunoprecipitates and bead-bound proteins were resolved on an 8% polyacrylamide gel. Molecular mass markers are indicated on the left. (B) Protein-binding assays with radiolabeled human MCM7n protein. Rabbit reticulocyte lysates programmed with human MCM7n cRNA were incubated with [^{35}S]methionine, and equivalent amounts of radiolabeled proteins were incubated with rabbit anti-MCM7n antiserum (αMCM7n) or were incubated with GST-FSH15, GST-Rb, GST-Rb Δ89-140, GST-Rb Δ128-167, GST-Rb Δ249-309, GST-Rb Δ309-343, GST-Rb Δ343-389, GST-p107, and GST-p130 fusion proteins. Immunoprecipitates and bead-bound proteins were resolved on an 8% polyacrylamide gel. Molecular mass markers are indicated on the left.

sufficient to mediate Rb-MCM7 interactions in vitro (data not shown). We conclude from these data that MCM7 can form a complex with full-length Rb in vitro and that these interactions are mediated by the Rb amino terminus and the carboxy terminus of MCM7.

In an attempt to further delineate portions of Rb required for interactions with MCM7, a series of GST-Rb fusion proteins carrying small deletions spanning nearly the entirety of the Rb amino terminus were examined for their capacity to bind MCM7n in vitro (69, 79). In a reciprocal assay, full-length Rb proteins carrying these amino-terminal internal deletions were examined in protein-binding assays with GST-MCM7n. As shown in Fig. 2B (center panel), there were no detectable differences in the capacity of wild-type and internally deleted Rb-fusion proteins to bind MCM7n in vitro. Identical results were obtained in the reciprocal assay (data not shown). One potential explanation for these results is that MCM7 interacts with portions of the Rb amino terminus that are larger than the deletions that we have examined. Indeed, similar results have been noted for physical interactions between the Rb amino terminus and p84, a nuclear matrix-associated protein (20). Alternatively, it is possible that Rb amino acids within two regions not deleted in these constructs, amino acids 1 to 88 and 167 to 249, are required for the formation of Rb-MCM7 complexes.

To determine if MCM7 can bind the amino termini of other Rb family members, the amino-terminal 418 and 387 amino acids of p130 and p107, respectively, were expressed as GST fusion proteins and were incubated with radiolabeled, in vitro-translated MCM7n protein. Equal amounts of each GST fusion protein were bound to glutathione-agarose beads and mixed with MCM7-containing reticulocyte extracts. As shown in Fig. 2B (right panel), MCM7n bound to the amino termini of each Rb family member with equal efficiency.

Rb associates with MCM7 in mammalian cells. To determine whether Rb and MCM7 associate in vivo, a sequential coimmunoprecipitation assay was performed. Human ML-1 cells were metabolically labeled with [^{35}S]methionine, nondenatured cell extracts were prepared, and lysates were immu-

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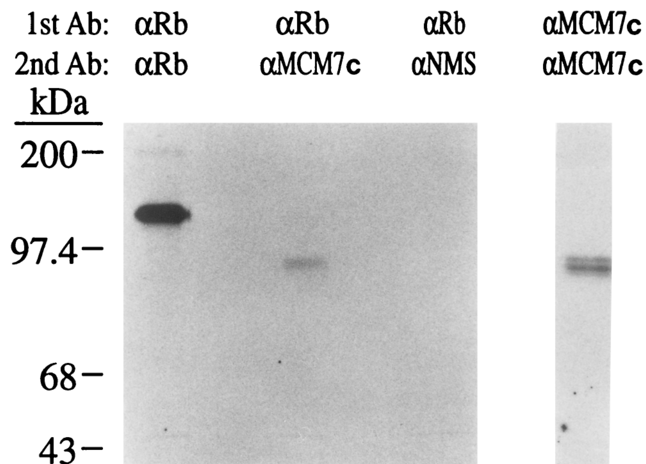

FIG. 3. Association of Rb and MCM7 proteins in human cells. ML-1 cells were metabolically labeled with [^{35}S]methionine, and nondenatured extracts were incubated with an anti-Rb monoclonal antibody (Ab [XZ77 or αRb]) or mouse anti-MCM7c antiserum (αMCM7c). The resulting immunoprecipitates were solubilized and reimmunoprecipitated with anti-Rb antibody (XZ77), mouse anti-MCM7c antiserum (αMCM7c), or normal mouse serum (αNMS), and precipitates were resolved on an 8% acrylamide gel. Molecular mass markers are indicated on the left.

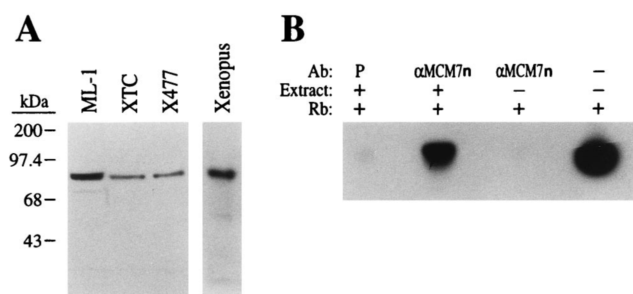

FIG. 4. Association of human Rb and *Xenopus* MCM7 proteins in *Xenopus* egg extracts. (A) Detection of *Xenopus* MCM7 protein via Western blotting. Denatured cell extracts prepared from human ML-1 cells, two *Xenopus* cell lines (XTC and X477), and *Xenopus* eggs (Xenopus) were resolved on an 8% acrylamide gel, transferred to nitrocellulose, and probed with mouse anti-MCM7n antiserum. Molecular mass markers are indicated on the left. (B) Association of the amino-terminal 400 amino acids of human Rb protein with *Xenopus* MCM7. A histidine-tagged amino-terminal Rb protein harvested from baculovirus-infected cells was mixed with *Xenopus* egg extracts and incubated with preimmune (P) or immune (αMCM7n) rabbit anti-MCM7n antiserum. Histidine-tagged Rb protein in buffer (far right lane) and MCM7 immunoprecipitates were resolved on an 8% acrylamide gel, and Rb protein was detected by Western blotting with a monoclonal anti-Rb antibody (Ab) that binds an amino-terminal epitope (C36 [85]).

noprecipitated with XZ77, a monoclonal antibody that binds an epitope within the Rb pocket (40). Following immunoprecipitation with XZ77, Rb precipitates were solubilized with SDS and denatured proteins were reimmunoprecipitated with antibodies against Rb, MCM7c, or control antibodies. As shown in Fig. 3 (left panel, left lane), secondary anti-Rb precipitates contained abundant amounts of Rb protein. MCM7c immune serum precipitated a characteristic 82- to 85-kDa protein doublet from primary immune complexes prepared with XZ77 (Fig. 3, left panel, center lane). This doublet is indistinguishable from proteins detected in a reimmunoprecipitation of immune complexes prepared with MCM7c antiserum (right panel). As expected, secondary precipitates prepared with control antiserum contained neither Rb or MCM7 proteins (Fig. 3, left panel, right lane). We conclude from these data that Rb and MCM7 proteins may be found in association with one another in mammalian cells.

The Rb amino terminus binds to *Xenopus* MCM7 and inhibits DNA replication. Since complexes between Rb and MCM7 proteins were detected in yeast, in vitro, and in mammalian cells it became of interest to pursue experiments designed to determine the functional consequence of Rb-MCM7 interactions. Given that Rb functions to negatively regulate cell cycle progression and enforce terminal differentiation, we hypothesized that Rb might negatively regulate MCM7 and in turn DNA replication. To explore this hypothesis, a *Xenopus* cell-free DNA replication system was employed (4–6, 41). *Xenopus* eggs are physiologically arrested in metaphase of meiosis. Upon lysis by centrifugation, calcium is released from internal stores, leading to cyclin degradation and transition of the extracts into interphase. Inclusion of cyclohexamide prevents de novo cyclin synthesis and cell cycle progression. Synthetic nuclei formed upon addition of demembranated sperm chromatin to these extracts will undergo a single, complete round of semiconservative DNA replication. cdk2 kinase activity is required to initiate DNA synthesis, since treatment of extracts with a cdk2 inhibitor, p21, prevents replication (3, 44, 76, 87). Additionally, extracts that have been immunodepleted for either cdk2 or cyclin E are also incapable of initiating DNA replication (44, 87).

At the outset, we wished to determine whether anti-human MCM7 antisera bound *Xenopus* MCM7 and whether the amino terminus of human Rb could associate with *Xenopus* MCM7 (XMCM7) in vitro. Previous studies have identified two proteins with molecular masses of 96 and 85 kDa that are bound by anti-XMCM7 antiserum in *Xenopus* extracts (72). To assess whether anti-human MCM7 serum bound its *Xenopus* homolog, whole-cell extracts were prepared from human and *Xenopus* cell lines, and *Xenopus* eggs and Western blots of each extract were probed with anti-MCM7n antiserum. As shown in Fig. 4A, a single prominent protein of 85 kDa was detected in each cell extract examined. Thus, the anti-human MCM7n antiserum that we have prepared appears not to bind the 96-kDa XMCM7 isoform. We then asked whether *Xenopus* MCM7 could associate with the amino terminus of human Rb by mixing *Xenopus* egg extracts with recombinant Rb protein and performing sequential immunoprecipitations and Western blots. As shown in Fig. 4B, Rb was detected only when mixtures of egg extracts and recombinant protein were immunoprecipitated with anti-MCM7n immune serum. Preimmune serum (Fig. 4B) did not precipitate detectable amounts of Rb, nor did immune serum in the absence of *Xenopus* egg extract. We conclude from these results that anti-human MCM7n antiserum binds a *Xenopus* protein of the expected size and that the amino terminus of human Rb can form complexes with *Xenopus* MCM7 in vitro.

Satisfied that Rb can associate with *Xenopus* MCM7, we wished to establish that the Rb amino terminus would not block DNA replication due to nonspecific effects, such as interactions that might preclude the formation of a nuclear envelope, block nuclear transport, or inhibit cdk2 kinase activity. To ensure that Rb did not interfere with the assembly of a nuclear envelope, demembranated sperm chromatins were mixed with *Xenopus* egg extracts and nuclear assembly was monitored over time by staining with Hoechst 33258. Parallel experiments were performed with the addition of an amino-terminal GST-Rb fusion protein or GST-FSH15, a control GST fusion protein. As shown in Fig. 5A, the inclusion of

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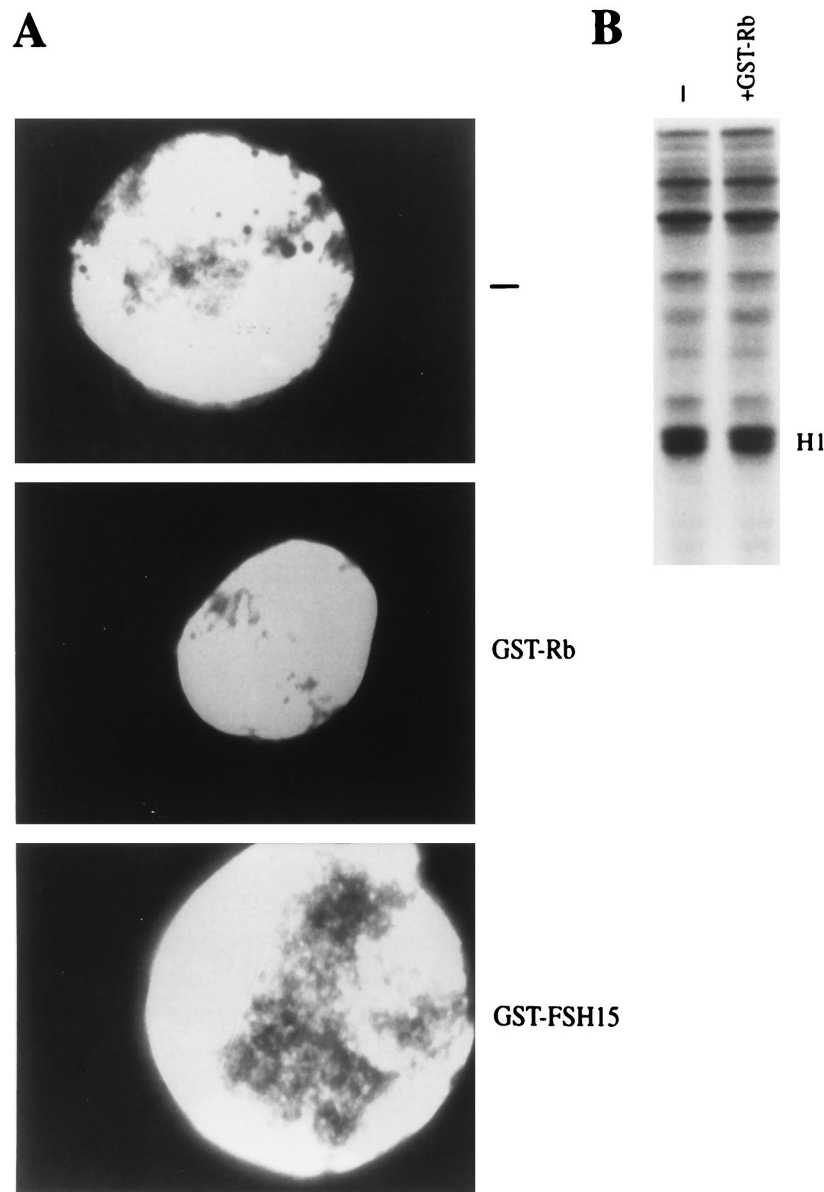

FIG. 5. Incubation of Rb protein with *Xenopus* egg extracts does not interfere with the formation of nuclei or cdk2 kinase activity. (A) Demembranated sperm chromatin was incubated in *Xenopus* interphase extracts (−) or extracts preincubated with an amino-terminal GST-Rb fusion protein (GST-Rb) or a control fusion protein (GST-FSH15). The formation of intact nuclei was assayed by staining with Hoechst 33258 and was visualized by fluorescence microscopy. Nuclei were somewhat variable in size, and representative examples are shown. (B) p13^{suc}-Sepharose beads were incubated with *Xenopus* egg extracts (−) or extracts that had been precleared by incubation with Rb protein (GST-Rb). Bead-bound kinase activity was detected in an in vitro kinase assay using histone H1 as substrate. The position of histone H1 is indicated on the right.

GST-Rb had little apparent effect on nuclear envelope assembly since the formation of nuclei in control and Rb-containing extracts was indistinguishable. Identical results were obtained at every additional time point examined (data not shown). To determine whether the inclusion of GST-Rb protein altered the capacity of *Xenopus* extracts to actively transport nuclear proteins, a rhodamine-labeled transport substrate was added to nuclei that had or had not been programmed with GST-Rb and the distribution of the substrate was analyzed by fluorescence microscopy (66). Both control and Rb-treated extracts were indistinguishable with respect to transport of the test substrate (data not shown). Taken together, these results indicate that the addition of the Rb amino terminus does not preclude the assembly or transport functions of nuclei in *Xenopus* egg extracts.

As mentioned previously, in conjunction with proper nuclear assembly cdk2 kinase activity is required to initiate DNA replication. To determine whether the amino terminus of Rb bound cdk2-associated kinases or otherwise interfered with cdk2 kinase activity, *Xenopus* egg extracts or extracts that had been precleared with an excess of GST-Rb were incubated with p13^{suc}-Sepharose beads. The vast majority of p13^{suc}-bound H1 kinase activity in *Xenopus* extracts has previously been shown to be directed by cdk2-associated kinases (26). The abundance of p13^{suc}-bound cdk2 was determined by Western blotting, and p13^{suc}-bound kinase activity was monitored via an in vitro

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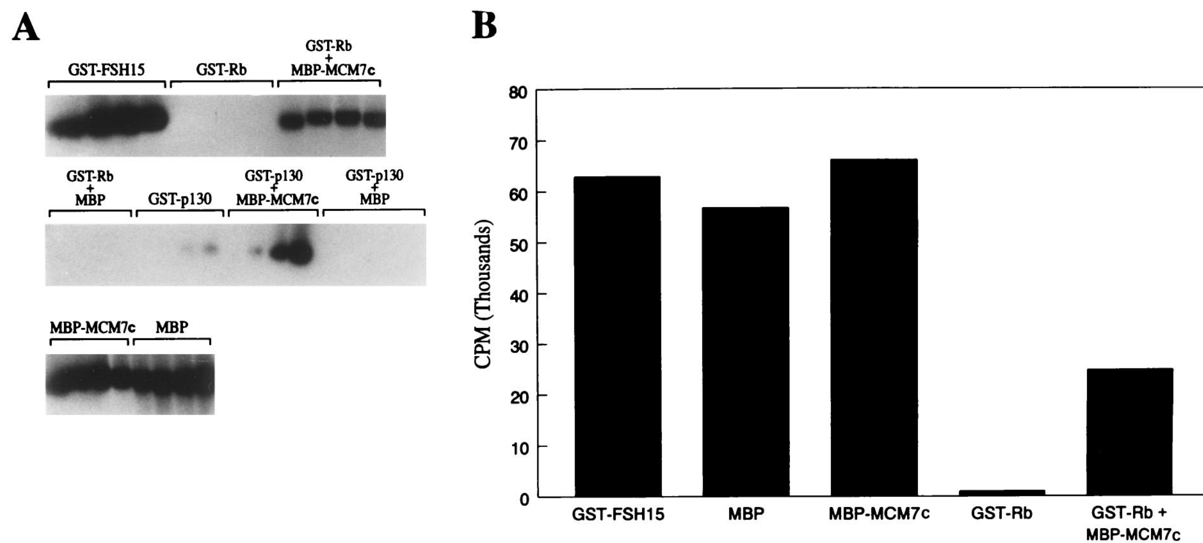

FIG. 6. Inhibition of in vitro DNA replication by Rb family members. (A) Replication of sperm chromatin in *Xenopus* egg extracts treated with the indicated fusion proteins was established by pulse-labeling via incorporation of [α-^{32}P]dCTP. For each extract, incorporation of [α-^{32}P]dCTP into DNA was monitored following 60, 90, 120, and 150 min of incubation. (B) Quantification of DNA replication at the 90-min time point shown in panel A. Replication assays were quantified by scanning in a PhosphorImager.

kinase assay with histone H1 as the substrate. Western blots of *Xenopus* extracts and extracts that were precleared with GST-Rb contained equivalent amounts of p13^{suc}-bound cdk2 (data not shown). Similarly, equivalent amounts of p13^{suc}-bound H1 kinase activity were harvested from *Xenopus* extracts or extracts precleared with GST-Rb (Fig. 5B). These results are in agreement with reports from our laboratory, indicating that the amino terminus of Rb does not bind a variety of cyclin-cdk kinases (78, 79). Instead, interactions of cdk kinases with Rb appear to be mediated via the Rb pocket (24, 34). We conclude that GST-Rb does not appreciably interact with *Xenopus* cdk2 or alter the abundance of H1 kinase activity in *Xenopus* egg extracts.

To determine whether inclusion of the Rb amino terminus in an in vitro replication assay perturbed the initiation or synthesis of DNA, we wished to examine DNA replication in egg extracts in the presence or absence of GST-Rb. Centrifugation of egg extracts produces cytosolic and membrane fractions that are amenable to long-term storage. Reconstitution of these fractions in the presence of sperm chromatin and an ATP-regenerating system provides for the formation of replication-competent nuclei. *Xenopus* egg extracts were incubated with GST-Rb or equivalent amounts of GST-FSH15, and replication of sperm chromatin was monitored over a 150-min time course via incorporation of [α-^{32}P]dCTP. As shown in Fig. 6, in vitro DNA replication occurred throughout the time course examined in the presence of GST-FSH15. In contrast, the replicative capacity of *Xenopus* extracts incubated with GST-Rb was abolished in parallel assays. To determine if the inhibition of DNA replication by the amino terminus of Rb would extend to other Rb family members, a GST-fusion protein prepared with the amino terminus of p130 was incubated with *Xenopus* egg extracts and in vitro DNA replication was quantified over the same time course. Similar to results with Rb, inclusion of GST-p130 led to a marked reduction in DNA replication. Indeed, significant amounts of DNA replication in the presence of GST-p130 were noted only at the 120- and 150-min time points (Fig. 6).

Although we had previously established that the amino termini of Rb family members bind MCM7 in vitro and that complexes of Rb and MCM7 may be detected in vivo and in *Xenopus* extracts, we wished to corroborate that the inhibition of in vitro DNA replication by Rb family members was due to the binding of MCM7. Should the amino termini of Rb and p130 block replication via a physical interaction with MCM7, we reasoned that the addition of MCM7c, the carboxy-terminal portion of MCM7 that is sufficient for Rb-MCM7 interactions, to *Xenopus* extracts should prevent Rb family members from inhibiting DNA replication. Thus, GST-Rb and GST-p130 proteins were preincubated with MCM7c expressed as a fusion with MBP or MBP alone, and these mixtures were added to *Xenopus* extracts. As shown in Fig. 6, preincubation of GST-Rb and GST-p130 proteins with MBP did little to reverse their inhibition of DNA replication. In contrast, preincubation of GST-Rb and GST-p130 proteins with MBP-MCM7c restored the replicative capacity of *Xenopus* egg extracts. Incubation of egg extracts with MBP or MBP-MCM7c alone had no effect on the replication of DNA (Fig. 6).

DISCUSSION

Nearly 25 years ago, the restriction point was defined as a period in late G_1 subsequent to which a mammalian cell was committed to the replication of its genome (68, 83). The Rb protein is widely believed to function as a central arbiter of this checkpoint, ensuring the orderly progression of cells into S phase. A large body of evidence indicates that Rb indirectly controls the rate of cell proliferation via its physical or functional interaction with a constellation of transcription factors, including E2F, Elf-1, Sp1/Sp3, myoD, C/EBP, and ATF-2 (for a review, see reference 37). Such interactions are believed to negatively regulate the transcription of genes required for cell cycle progression and stimulate genes associated with growth inhibition and/or differentiation. Here, we report that Rb can also directly prevent cell cycle progression via its association with MCM7, an essential replication protein and a component of licensing factor.

Suggestions that Rb family members play a role in the neg-

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ative regulation of DNA replication spring from three previous lines of investigation. First, skeletal muscle cell lines derived from $Rb^{-/-}$ animals form multinucleated myotubes in vitro and yet are stimulated to synthesize DNA by serum (73). Consistent with these observations, recent studies with nullizygous animals have shown that (i) nuclei in $Rb^{-/-}$ myotubes show evidence of endoreduplication and are two to four times larger than those of their $Rb^{+/+}$ littermates and (ii) $Rb^{-/-}$ myotubes actively synthesize DNA in vivo (88). Should the deregulation of DNA replication in $Rb^{-/-}$ myotubes reflect in part the absence of Rb-MCM7 complexes, one must conclude that Rb-related proteins cannot completely compensate for the loss of this Rb function. In apparent accord with this prediction, inhibition of DNA replication in vitro by GST-p130 is reproducibly less efficient than parallel experiments performed with GST-Rb (Fig. 6). It will be of interest to determine if such differences extend to p107 by assessing its relative capacity to inhibit DNA replication. Second, antagonists of Rb family members, such as SV40 large-T antigen, induce unscheduled DNA synthesis and aneuploidy in certain cell types (27, 67, 71). Whether the binding of viral oncoproteins to Rb-family members results in their dissociation from MCM7 is currently under investigation. Finally, Rb has previously been reported to associate with Purα, a single-stranded DNA-binding protein that binds purine-rich sequences at replication origins (1, 2, 45). Rb binds to Purα in a phosphorylation-dependent manner via its carboxy-terminal pocket region, and thus it is possible that Rb forms a tripartite complex with MCM7 and Purα at origins of replication. Similarly, additional DNA-binding proteins sequestered within the pocket region of Rb-related proteins could be envisioned to participate in the targeting of MCM complexes to euchromatin.

The genome is replicated only once during each cell cycle in most eukaryotic cells, and many of the regulatory mechanisms governing the initiation of DNA replication have been identified by using cell extracts prepared from *Xenopus* eggs (3, 26, 44). DNA added to such extracts is assembled into functional nuclei that undergo a single round of semiconservative replication. To undergo a subsequent round of replication in vitro, either *Xenopus* nuclei have to progress through mitosis or the nuclear membrane must be transiently permeabilized. To account for these results, Blow and Lasky proposed that chromosomes are licensed to undergo DNA replication once during each cell cycle by a regulator termed replication licensing factor (RLF) (3, 5). Binding of RLF to chromatin was proposed to mark a replication origin for firing, and replication was thought to inactivate RLF, ensuring that a single round of DNA synthesis ensues. Following the dissolution of the nuclear envelope in mitosis, chromosomes were predicted to once again become licensed for replication via the binding of RLF to chromatin. Recently, RLF has been resolved into two components, both of which are required for DNA replication (9, 48, 57). One component appears to facilitate the association of RLF with chromatin, and the other is a protein complex that contains MCM2 through -7 (47, 81). MCM proteins were originally identified in *Saccharomyces cerevisiae* as functions necessary for the maintenance of minichromosomes carrying certain types of autonomously replicating sequences (58, 86). Such functions have been well conserved throughout evolution, since MCM genes have been isolated from a wide variety of eukaryotes, including plants, insects, amphibians, and mammals (10, 82). MCM proteins are tightly associated with chromatin until replication begins, whereupon they are released into the nucleoplasm and rebound to chromatin during mitosis. Indeed, this fluctuation in their subcellular localization provided the first indication that MCM proteins might function as a component of RLF.

Although MCM proteins have been identified as components of RLF, questions regarding the regulation of RLF function remain unresolved. For example, what prevents chromatin-associated MCM complexes from triggering DNA replication at inappropriate times? A possibility suggested by results reported here is that the activity of MCM complexes are regulated via their association with Rb family members. This association with MCM7 is mediated by the amino termini of Rb family members, a relatively unexplored portion of these proteins that has previously been shown to be unnecessary for interactions with transcription factors such as E2F (37). Interestingly, the association of Rb-related proteins with MCM7 requires a portion of MCM7, the carboxy-terminal 137 amino acids, that is not conserved among MCM family members. Thus, Rb, p107, and p130 appear to have evolved to interact specifically with one component of RLF. Although we have shown that Rb interacts with MCM7 in a yeast two-hybrid screen, in in vitro protein-binding assays, in mammalian cells, and in *Xenopus* extracts, it is worth noting that our experiments do not prove that these interactions are direct. It remains formally possible that one or more evolutionarily conserved proteins function as a bridge between Rb family members and MCM7. Additionally, our experiments do not address whether Rb family members prevent MCM7 from joining RLF or whether Rb family members are tethered to RLF via their association with MCM7. Since MCM7 is invariably found associated with other MCM proteins in vivo, the latter possibility may be more likely (28, 72, 74).

Inclusion of GST-Rb or p130 fusion proteins in *Xenopus* extracts resulted in a marked reduction in DNA replication. Importantly, this negative regulation of in vitro DNA replication appears to be MCM7 dependent, since preincubation of Rb and p130 with the carboxy-terminal 137 amino acids of MCM7 (MBP-MCM7c) restored the replicative capacity of *Xenopus* extracts. At least two possible mechanisms may account for the capacity of MCM7c to restore DNA replication. First, MCM7c could supplant the loss of XMCM7 function by directly interacting with RLF. Although this is a formal possibility, it is unlikely, since the amino-terminal 122 amino acids of MCM7 are required for interactions with components of licensing factor (72). A more likely scenario is that preincubation of Rb and p130 with MCM7c saturated MCM7 binding sites, thereby preventing their interaction with XMCM7. It is worth noting that our results do not enable us to formally rule out the possibility that inclusion of MCM7c prevents the interaction of Rb and p130 with yet another *Xenopus* protein required for replication. Regardless of the precise mechanism of rescue, results from in vitro replication assays strongly support the idea that Rb family members can negatively regulate DNA replication. Taken together with in vitro and in vivo evidence of protein complexes between Rb, p107, or p130 and MCM7, we conclude that MCM7, and by extension RLF, is a target of tumor suppressor gene function. Interestingly, the product of yet another tumor suppressor gene, p53, has been reported to negatively regulate the initiation of DNA replication in vitro (13, 21). Whether MCM7 function is directly or indirectly influenced by p53 has yet to be established.

Given the biochemical and functional results reported here, we hypothesize that the initiation of DNA replication is prevented, at least in part, by the association of Rb family members with MCM7 in G_1. Rb, p107, and p130 are phosphorylated by cyclin-cdk kinases as cells approach the G_1/S transition, and we speculate that phosphorylation may trigger the release of MCM7 (83). Since *Xenopus* extracts carry active cyclin-cdk

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kinases, why do Rb and p130 proteins block in vitro DNA replication? As we have previously reported, the Rb amino terminus is not bound by cyclin-cdk kinases and is only marginally phosphorylated by such complexes in vitro (78). Indeed, interactions of Rb family members with cyclin-cdk kinases requires their respective pocket regions, a domain not included in the fusion proteins that we have examined (17, 24). As the cell cycle progresses, dephosphorylation of Rb family members in anaphase may facilitate their reassociation with MCM7 and inhibition of DNA replication until passage through the restriction point during the subsequent cell cycle. Experiments to determine the abundance of complexes between Rb family members and MCM7 as a function of cell cycle progression are currently under way.

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