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Complete In Vitro Reconstitution of Adeno-Associated Virus DNA Replication Requires the Minichromosome Maintenance Complex Proteins[▽]

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Adeno-associated virus (AAV) replicates its DNA exclusively by a leading-strand DNA replication mechanism and requires coinfection with a helper virus, such as adenovirus, to achieve a productive infection. In previous work, we described an in vitro AAV replication assay that required the AAV terminal repeats (the origins for DNA replication), the AAV Rep protein (the origin binding protein), and an adenovirus-infected crude extract. Fractionation of these crude extracts identified replication factor C (RFC), proliferating cell nuclear antigen (PCNA), and polymerase δ as cellular enzymes that were essential for AAV DNA replication in vitro. Here we identify the remaining factor that is necessary as the minichromosome maintenance (MCM) complex, a cellular helicase complex that is believed to be the replicative helicase for eukaryotic chromosomes. Thus, polymerase δ, RFC, PCNA, and the MCM complex, along with the virally encoded Rep protein, constitute the minimal protein complexes required to reconstitute efficient AAV DNA replication in vitro. Interfering RNAs targeted to MCM and polymerase δ inhibited AAV DNA replication in vivo, suggesting that one or more components of the MCM complex and polymerase δ play an essential role in AAV DNA replication in vivo as well as in vitro. Our reconstituted in vitro DNA replication system is consistent with the current genetic information about AAV DNA replication. The use of highly conserved cellular replication enzymes may explain why AAV is capable of productive infection in a wide variety of species with several different families of helper viruses.

Adeno-associated virus (AAV) is a single-stranded DNA (ssDNA) virus belonging to the parvovirus family that is commonly found as a contaminant of human adenovirus (Ad) isolates in the wild (for a review, see reference 3). AAV requires a helper virus for efficient DNA replication and viral propagation and in the absence of a helper virus produces little AAV gene expression and virtually no DNA amplification. The Ad genes that have helper function for AAV are E1a, E1b, E4 orf 6, VA, and E2a. With the exception of the E2a DNA binding protein (DBP), none of these genes code for enzymes that are directly involved in DNA replication, and an Ad deletion of E2a has only a modest (<5-fold) effect on AAV DNA replication in vivo (7). Thus, in the presence of Ad coinfection, AAV relies primarily on cellular enzymes for DNA replication.

AAV replicates by a strand displacement method using a hairpinned terminal repeat (TR) as a primer (Fig. 1) (3). The ssDNA genome uses the hairpin primer to synthesize a duplex DNA molecule that is covalently closed at one end by the hairpin structure. This hairpin is then resolved to give a linear double-stranded DNA (dsDNA) molecule in a process called terminal resolution. During resolution, the TR is cleaved at a unique site on one strand (the terminal resolution site [*trs*]), and the hairpinned TR is repaired to make an open-ended duplex intermediate. The repaired TR is then denatured and reannealed in a process called reinitiation to form a double-hairpinned intermediate that initiates a round of strand displacement synthesis. Strand displacement synthesis from the dsDNA intermediate then generates a single-stranded genome that is packaged. The virus-encoded Rep78 and Rep68 proteins have been shown to have the site-specific DNA helicase and endonuclease activities required to carry out both terminal resolution and reinitiation in vitro (13, 23, 37).

We and others have developed in vitro AAV DNA replication assays that faithfully recapitulate most aspects of AAV DNA synthesis in vivo and produce full-length AAV DNA de novo (19, 31). These assays use linear AAV DNA that contains either open or covalently closed terminal repeats at both ends and require the presence of both the AAV terminal repeat (the AAV ori) and Rep78 or Rep68 enzymes for efficient DNA synthesis (Fig. 1). These studies (18, 19, 31) have also shown that all of the intermediates seen in vivo during AAV DNA replication are recapitulated in vitro and that extracts from Ad-infected cells are much more active than uninfected extracts (>50-fold).

Fractionation of crude cell lysates from Ad-infected and uninfected HEK293 cells allowed us to identify three cellular complexes that were essential for in vitro AAV DNA replication (17, 18). The three complexes, polymerase δ (Pol δ), replication factor C (RFC), and proliferating cell nuclear antigen (PCNA) (Fig. 2, lane 2), are all involved in cellular DNA replication and were shown previously to be involved in leading-strand synthesis of simian virus 40 (SV40) DNA (30). As expected, Pol α was not required for AAV DNA synthesis, nor

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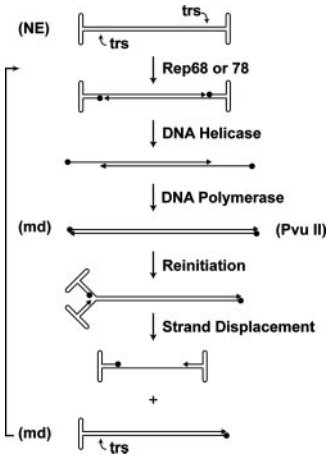

FIG. 1. Mechanism of AAV DNA replication. The scheme illustrates the key steps during AAV DNA replication with No-End DNA substrate (NE). The alternative linear dsDNA substrate generated from PvuII digestion of psub201 is also shown (Pvu II). The black dot represents the 5′ ends, horizontal arrows indicate 3′ ends, and *trs* indicates the AAV terminal resolution site. Note that the monomer duplex (md) intermediate exists in two forms, with and without a covalently closed hairpinned end. See the text for more details.

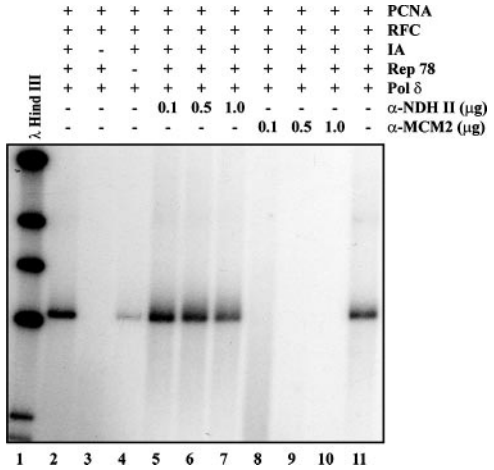

FIG. 2. In vitro DNA replication with partially purified fraction IA in the presence of polyclonal MCM2 antibody (α-MCM2). Standard replication reactions (15 μl) were performed and contained, where indicated, RFC (0.01 μg), Rep78 (0.2 μg), PCNA (0.4 μg), IA (6 μg), Pol δ (0.4 μg), and/or MCM2 antibody (0.1 to 1.0 μg). Replication products were subjected to DpnI digestion prior to electrophoresis.

was Pol ε (17). In addition, antibody inhibition studies of uninfected crude extracts suggested that in vitro AAV DNA replication may require the cellular single-stranded DNA binding protein replication factor A (RPA) (18), and supplementation of uninfected extracts with Ad DBP suggested that in vitro AAV replication was stimulated by Ad DBP approximately two- to fourfold (32). These results suggested that there may be a requirement for one or more single-stranded DNA binding proteins. Finally, cell fractionation studies of Ad-infected extracts showed that AAV DNA synthesis also required an unidentified factor(s) in a partially purified fraction we termed P-cell IA (17). Our attempts to purify this missing factor failed, suggesting either that the factor was unstable or that it consisted of a complex that fractionated into multiple parts upon further purification. Since previous work (19, 31) had shown that the missing component was likely to be involved in strand elongation, we focused on DNA helicases and other DNA-related enzymes. Using antibodies, purified recombinant proteins, and some protein inhibitors, we examined the possible involvement of a variety of enzymes that were present in fraction IA. Some of the proteins examined included the BLM helicase, the WRN helicase, high-mobility group proteins 1 and 2, NDH II, and Cdk1. None of these appeared to be necessary for AAV DNA replication, nor could they substitute for fraction IA (17). Here we present evidence that one or more proteins of the MCM complex could substitute for the previously unidentified component, and we demonstrate that we can completely reconstitute AAV DNA replication in vitro with purified Rep78, MCM, Pol δ, RFC, and PCNA.

MATERIALS AND METHODS

Reagents. Western blotting detection reagents were purchased from Millipore. [α-^{32}P]dATP (3,000 Ci/mmol) and [α-^{3}H]TTP were purchased from Perkin Elmer. Phosphocellulose (P11) and DE 52 cellulose were acquired from Whatman. Phenyl-Sepharose, protein A and G Sepharose, Q-Sepharose, poly(dA), and oligo(dT) were purchased from Amersham. Nickel-nitrilotriacetic acid (Ni-NTA) agarose was purchased from Qiagen. Nucleotides and salmon type III DNA were obtained from Sigma. HindIII, DpnI, and λ DNA were purchased from New England Biolabs. Antibodies to MCM2 to -7 were purchased from Bethyl.

HEK293 lysate fractions. HEK293 cell extracts were generated as described by Nash et al. (17). Fraction IA was obtained as described previously by Nash et al. (17). Briefly, Ad-infected crude extract was fractionated on phosphocellulose equilibrated with buffer A (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% glycerol, 0.01% NP-40, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A) containing 0.2 M NaCl before the extract was loaded. The flowthrough fraction was designated fraction I. Fraction I was further purified on a Q-Sepharose column preequilibrated with buffer A containing 0.1 M NaCl. The flowthrough (fraction IA) was collected. Fraction I of CHO-K and xrs-5 cells was generated using crude cell extract from these cell lines and fractionated on phosphocellulose equilibrated with buffer A, and the flowthrough was collected.

Replication proteins. Baculovirus-expressed AAV Rep78 was purified as previously described (18). Baculovirus-expressed RPA subunits and Pol δ subunits were purified as described by Nash et al. (17). PCNA was purified from a bacterial expression clone, as described by Fien and Stillman (11). RFC was purified as described by Tsurimoto and Stillman (27).

Immunodepletion of MCM2 from fraction IA was performed by incubating MCM2 antibody (5 μg) with 100 μl of fraction IA (2 mg/ml) for 2 h at 4°C, followed by the addition of protein A agarose (50 μl slurry) and a further incubation overnight at 4°C. A control with fraction IA was performed under identical conditions but in the absence of antibody. The agarose was precipitated by centrifugation at 5,000 rpm for 2 min. The supernatant was removed, and the agarose was washed two times with 200 μl of 50 mM Tris (pH 7.5) buffer.

MCM purification. MCM2 (TC117321) and MCM6 (TC110945) cDNA clones were purchased from Origene. A histidine tag was added to the N terminus using PCR and the primers GAGGAATTCCCACCATGCATCATCACCATCACCA CATGGCGGAATCATCGGAATCCTTC and CGTTGTGGCCGTGGCTGT CGAC for MCM2 and GAGCTGCAGCCACCATGCATCATCACCATCACC ACATGGACCTCGCGGCGGCAGCGGAG and GCTAAGCTTGGAGACG TCAGGCAC for MCM6. For purification of the MCM complex, typically MCM2 or MCM6 His-tagged plasmids (30 μg per 15-cm dish) were transfected into 10- by 15-cm dishes of HEK293 cells using TransIt for 293 cells (Miris). The cells were harvested by scraping 48 h posttransfection and centrifugation. The cells were lysed by Dounce homogenization in buffer A, and then NaCl was added to a 0.2 M concentration and the lysate incubated on ice for 30 min. The lysate was centrifuged for 15 mins at 10,000 × *g* and the supernatant retained. The soluble lysate was fractionated on phosphocellulose resin as described for 293 cell extracts above. The flowthrough material was collected and placed on a Ni-NTA agarose (Qiagen) equilibrated with buffer B (50 mM potassium phosphate, 10% glycerol, 0.01% NP-40, 15 mM imidazole, pH 7.0). The MCM complex was eluted with buffer B containing 0.2 M imidizole. The peak fraction

was dialyzed overnight with 25 mM Tris, 20% glycerol, 0.01% NP-40, and 50 mM NaCl, pH 7.5. The MCM complex was further purified on a Mono Q column equilibrated with 25 mM Tris, 20% glycerol, 0.01% NP-40, and 50 mM NaCl, pH 7.5, and eluted with a gradient of 0.05 to 1.0 M NaCl. Fractions positive for the MCM protein by Western blotting with MCM6 antibody were pooled.

The Mono Q MCM protein sample was characterized by tandem mass spectroscopy (MS/MS). A sample of the Mono Q MCM fraction was digested with trypsin and then loaded onto an LC Packing C18 Pep Map high-performance liquid chromatography column for liquid chromatography-MS/MS. Liquid chromatography-MS/MS analysis was carried out in the University of Florida Interinstitutional Center for Biotechnology Research using a hybrid quadrupole–time-of-flight mass spectrometer (QSTAR; Applied Biosystems, Framingham, MA). All MS/MS samples were analyzed using Mascot and the NCBInr database.

AAV DNA replication assay. No-End substrate was prepared as previously described by Snyder et al. (23) (Fig. 1). The standard replication reaction was carried out as previously described by Ni et al. (18, 19), and the mixture contained the following in a 15-μl volume: 30 mM HEPES, 7 mM MgCl2, 0.5 mM DTT, 4 mM ATP, 6 μCi [α-^{32}P]dATP, 100 μM deoxynucleoside triphosphate, 40 mM creatine phosphate, 33 ng creatine phosphokinase, 0.05 μg No-End, and 0.1 to 1.0 μg Rep 78 and crude extract or purified replication proteins. Reactions were incubated for 3 h at 37°C and then stopped with 35 μl of stop solution (0.3% sodium dodecyl sulfate [SDS], 17 mM EDTA, 0.7 μg/ml proteinase K). The samples were extracted with phenol-chloroform and precipitated with ethanol. The DNA was digested with DpnI for 1 h at 37°C and fractionated on a 0.8% agarose gel to separate unreplicated or partially replicated AAV DNA from completely replicated full-length duplex DNA. The gels were dried and exposed to X-ray film and/or a phosphorimager screen. Phosphorimage analysis was performed using a Amersham Typhoon 9200 phosphorimager. Only full-length DpnI-resistant DNA was counted as a replication product.

Polymerase assays. The reaction mixture contained the following in a 15-μl volume: 30 mM HEPES, 7 mM $MgCl_2$, 10% glycerol, 0.5 mM DTT, 4 mM ATP, 30 ng poly(dA)/oligo(dT) (A-T ratio, 1:1; Pharmacia), 0.75 μCi [^{3}H]dTTP, and 1 to 20 μg of DNA Pol δ. Reaction mixtures were incubated at 37°C for 30 min before the reaction was stopped by chilling on ice. An aliquot (5 to 8 μl) was spotted onto DE-81 paper, washed, and quantified in a scintillation counter. In some cases, activated salmon sperm DNA (50 ng DNA per reaction; Pharmacia) was used as a substrate in place of poly(dA)/oligo(dT). One unit of polymerase activity equals 1 nmol of [^{3}H]dTMP incorporated per hour.

siRNAs. Three targets (each) for MCM2, MCM6, and Pol δ were tested in a 24-well format. Twenty picomoles of Stealth small interfering RNA (siRNA) (from Invitrogen), 200 ng of pXX6, which expresses the Ad helper functions, and 200 ng of pSM620 (21), the infectious wild-type AAV plasmid, were transfected into HEK293 cells using 1.5 μl of Lipofectamine 2000 (Invitrogen) in 100 μl of OptiMEM medium. Cells were harvested at 24 h, and low-molecular-weight DNA was extracted by Hirt precipitation and quantitated by Southern blotting using a ^{32}P-labeled probe for AAV DNA. The probe was generated using the pSM620 plasmid, random DNA oligonucleotides, and Klenow fragment DNA polymerase. The amount of DpnI-resistant monomer duplex DNA was quantitated by phosphorimage analysis and compared to standard concentrations of pSM620 plasmid DNA. Primer names of Stealth interfering RNA from Invitrogen were as follows: MCM2HSS106390, MCM2HSS106391, and MCM2HSS106392; MCM6HSS106402, MCM6HSS106403, and MCM6HSS106404; and POLD1HSS108211, POLD1HSS108212, and POLD1HSS108213.

RESULTS

Antibodies to MCM2 completely inhibit AAV DNA replication. Recent evidence indicated that the MCM complex is likely to be the eukaryotic replicative helicase (see reference 26 for a review) and therefore a potential candidate for AAV DNA replication. Addition of an anti-MCM2 antibody to an in vitro AAV DNA replication assay containing fraction IA resulted in complete inhibition of replication activity (Fig. 2). In contrast, an antibody to NDH II helicase had no effect on DNA replication (Fig. 2). Examination of fraction IA with anti-MCM2 antibody showed the presence of MCM2 in fraction IA (Fig. 3A). Further examination of fraction IA by Western analysis also showed the presence of the other members of

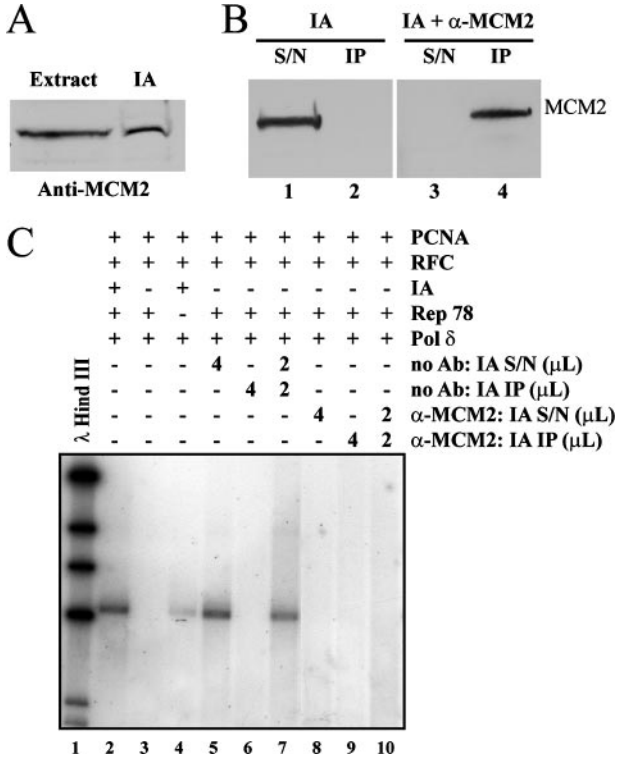

FIG. 3. (A) Western blot of a crude Ad-infected cell extract and the partially purified fraction IA using anti-MCM2 antibody. (B) Immunodepletion of MCM from fraction IA with MCM2 antibody. Western blot using MCM2 antibody of the supernatant (S/N) or precipitate (IP) of fraction IA in the absence of antibody (left panel) or the presence of anti-MCM2 antibody (right panel). (C) In vitro DNA replication with the mock-treated or anti-MCM2 antibody immunodepleted fraction of IA shown in panel B. The supernatant after precipitation in the absence of antibody (no Ab: IA S/N) or in the presence of anti-MCM2 antibody (α-MCM2: IA S/N) was tested, along with the precipitant without antibody added (no Ab: IA IP) or with MCM2 antibody added (α-MCM2: IA IP). Standard replication reactions were carried out, and mixtures contained, where indicated, RFC (0.01 μg), Rep78 (0.2 μg), PCNA (0.4 μg), IA (6 μg), and/or Pol δ (0.4 μg). Replication products were subjected to DpnI digestion prior to electrophoresis.

the MCM complex (MCM3 to -7) and the associated proteins CDC45, cdt1, and cdc6 (data not shown). Finally, immunodepletion of MCM2 from fraction IA with anti-MCM2 antibody (Fig. 3B) resulted in a complete loss of in vitro DNA replication (Fig. 3C, compare lanes 5 and 8). Because the MCM2 antibody is a neutralizing antibody, addition of the immunoprecipitated MCM complex (Fig. 3C, lanes 9 and 10) showed no replication activity.

Affinity-purified MCM complexes reconstitute AAV DNA replication. To purify the MCM complex, we overexpressed either His-tagged MCM2 or MCM6 proteins in HEK293 cells under a cytomegalovirus promoter and purified the MCM complexes by chromatography on phosphocellulose and Ni-NTA agarose columns (see Methods). The affinity-purified complexes obtained by using either His-tagged MCM2 or His-tagged MCM6 were both able to reconstitute AAV DNA replication in vitro (Fig. 4). We subsequently further purified the complex containing the His-tagged MCM6 protein by chroma-

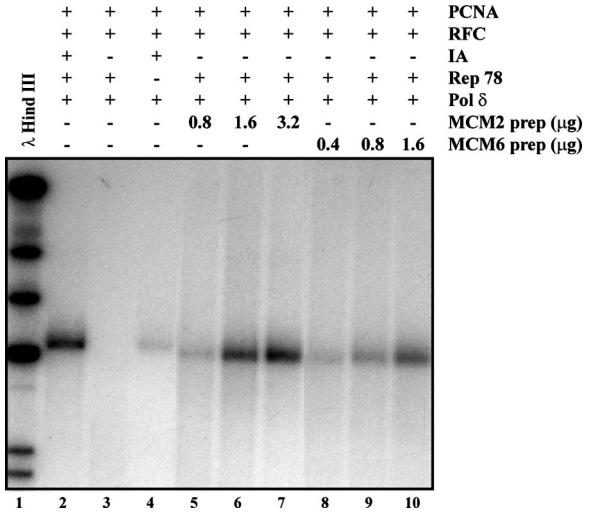

FIG. 4. In vitro DNA replication with purified MCM complexes. The MCM2 preparation was purified using the MCM2 His-tagged recombinant protein, and the MCM6 preparation was purified using the MCM6 His-tagged recombinant protein. Both MCM protein samples were purified by phosphocellulose and Ni-NTA chromatography as described in Methods for MCM purification. Standard replication reactions (15 μl) were carried out, and mixtures contained, where indicated, RFC (0.01 μg), Rep78 (0.2 μg), PCNA (0.4 μg), IA (6 μg), and/or Pol δ (0.4 μg). The replication products were digested with DpnI prior to electrophoresis.

tography on Mono Q as described in Methods. The Mono Q-purified MCM6-His-tagged complex was also able to substitute for fraction IA in the in vitro AAV DNA replication assay to reconstitute full activity (Fig. 5C) when it was combined with purified Rep, PCNA, RFC, and Pol δ. Notably, the level of the in vitro DNA replication activity that was seen in the reconstituted system using purified components was comparable to that seen with Ad-infected crude extracts and with assays that used the P-cell 1A fraction (Fig. 5C, compare lanes 2, 4, and 8; Fig. 4, compare lanes 2, 7, and 10).

Silver staining of SDS-polyacrylamide gels of the Mono Q MCM6 affinity-purified fraction revealed bands whose molecular weights were consistent with those of MCM2 to -7. In addition, several other bands were present (Fig. 5A). Western blotting confirmed the presence of the MCM2 to -7 proteins in the Mono Q-purified MCM6 His-tagged complex (Fig. 5B). In addition, Western analysis demonstrated that the purified MCM2 to -7 preparation contained α-tubulin, a small amount of cdc45, Cdt1, MCM10, and Smc1, and at least three other protein bands that were not identified. Tubulin is not expected to have a role in AAV DNA replication, but cdc45, Cdt1, MCM10, and Smc1 are known to be associated with the active MCM2 to -7 complex during eukaryotic chromosome replication (reviewed in references 1, 4, and 16). Although these proteins could be detected by Western analysis, with the possible exception of Cdt1, none of these other proteins were present in the same molar amounts as the MCM2 to -7 proteins as judged by the intensities of their bands in silver-stained gels. Additionally, GINS proteins, which have also been shown to be part of the active MCM complex, were not detected in the preparation by silver staining (GINS proteins are reviewed in references 1 and 15). We note also that the MCM6 affinity-purified Mono Q fraction shown in Fig. 5 was subjected to mass spectroscopy to confirm the identity of the proteins present in the fraction, as described in Methods. MCM2, -3, -4, -6, and -7 and tubulin were found to be present in the fraction. The other MCM complex-associated proteins discussed above

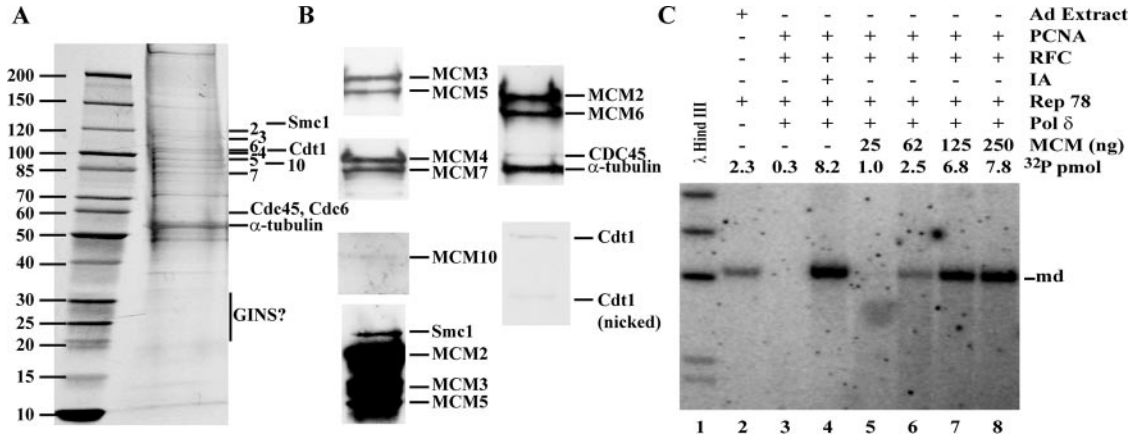

FIG. 5. (A) Silver stain from SDS-PAGE of the purified MCM6 His-tagged complex. The fraction shown was purified by phosphocellulose, Ni-NTA agarose, and Mono Q column chromatography as described in Methods. The positions of the MCM proteins are indicated by their appropriate numbers, 2 to 7 and 10. The positions of proteins commonly associated with the MCM complex are also indicated. Only some of the MCM or associated proteins were seen by silver staining. The left lane contains protein molecular weight markers. (B) Western blots of SDS-PAGE of the Mono Q fraction shown in panel A were blotted with antibodies as indicated, using the Milipore Immobilon reagent. In most cases one blot was sequentially examined with more than one antibody when the proteins were sufficiently separated on the gel. Panel B shows the relevant portions of six separate Western blots and in each case indicates the antibodies that were used to detect MCM-related proteins. (C) In vitro DNA replication with His-tagged MCM6 Mono Q-purified MCM complex and other purified DNA replication proteins. Standard replication reactions (15 μl) were carried out, and mixtures contained, where indicated, crude extract (60 μg), RFC (0.01 μg), Rep78 (0.2 μg), PCNA (0.4 μg), fraction IA (6 μg), Pol δ (0.4 μg), and/or the MCM complex (25 to 250 ng). Replication products were subjected to DpnI digestion prior to electrophoresis. The amount of ^{32}P nucleotide incorporated into monomer duplex AAV DNA was determined by phosphorimager analysis and is indicated for each lane.

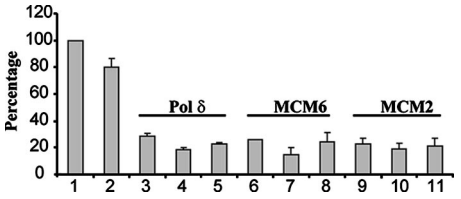

FIG. 6. Level of AAV DNA replication in HEK293 cells in the presence of siRNAs. Three targets (each) for MCM2, MCM6, and Pol δ were tested in a 24-well format. Twenty picomoles of siRNA, 200 ng of pXX6 (which expresses the Ad helper functions), and 200 ng pSM620 (infectious wild-type AAV plasmid) were transfected into 293 cells. Cells were harvested at 24 h, and low-molecular-weight DNA was extracted by Hirt precipitation and quantitated by Southern blotting using a ^{32}P-labeled AAV DNA probe. The amount of DpnI-resistant monomer duplex DNA was quantitated by phosphorimaging analysis and compared to standard concentrations of pSM620 plasmid DNA. 1, no siRNA; 2, control siRNA (no target); 3 to 5, siRNA for Pol δ; 6 to 8, siRNA for MCM6; 9 to 11, siRNA for MCM2. The average from four independent reactions is shown; error bars indicate standard deviations.

were not present in sufficient quantity to be detected by this method (data not shown).

siRNA targeted to MCM inhibits AAV DNA replication in vivo. To determine whether the MCM complex was also essential for AAV DNA replication in vivo, we isolated siRNAs targeted to MCM2 and MCM6 and tested their effect on AAV DNA replication in vivo. We included a negative control siRNA and siRNAs targeted to Pol δ as a positive control for replication inhibition, since we had previously shown Pol δ to be essential for AAV DNA replication in vitro (17). HEK293 cells were transfected with the wild-type AAV plasmid pSM620 (21) and the Ad helper plasmid, pXX6 (36), which expresses the Ad helper genes, along with the appropriate siRNA. All six siRNAs for MCM2 and MCM6 significantly reduced the level of AAV DNA replication (Fig. 6, compare lanes 6 to 11 with lane 1). Furthermore, the level of inhibition of AAV DNA replication seen with siRNA targeted to the MCM complex was comparable to that seen with siRNA targeted to Pol δ. In contrast, control siRNA had no significant effect on AAV DNA replication. We concluded that the MCM complex or some portion of it was essential for AAV DNA replication both in vivo and in vitro. These results also demonstrated that Pol δ is the essential DNA polymerase for AAV DNA replication in vivo.

DISCUSSION

Our results demonstrate that AAV DNA replication can be reconstituted in vitro with five purified protein complexes: Rep78 or -68, RFC, PCNA, Pol δ, and MCM. The level of DNA replication seen with these purified complexes is comparable to that seen with Ad-infected crude extracts and with the purified proteins plus fraction IA that we previously characterized (17, 19). It is worth noting that we are observing full-length AAV genome replication in our assay, using templates that initially have no free ends. In previous work, we demonstrated that the in vitro replication assay used here produces hybrid-density DNA when characterized on CsCl gradients after incorporaton of BrdUTP, indicating that at least one complete round of DNA replication had occurred (19). Additionally, the replication products were all digested with DpnI following replication and prior to gel electrophoresis and quantitation. Since DpnI will digest only methylated DNA strands, the AAV DNA observed by gel electrophoresis indicates that at least one round of full-length DNA replication has occurred. If partial DNA replication had occurred, we would have expected to see DpnI digestion products in the range of 5 to 2,100 bp, with most falling between 100 and 450 bp (13 DpnI sites are present in the AAV template).

Because parvoviruses provide their own DNA primer in the form of a hairpinned TR, they replicate their DNA by a mechanism that exclusively uses leading-strand DNA synthesis. Previous work with SV40 replication identified Pol δ as the leading-strand DNA polymerase and PCNA and RFC as essential accessory proteins (29, 30). This is consistent with our findings with AAV DNA replication. AAV is thus the third mammalian DNA viral system, after Ad and SV40, which has been completely reconstituted in vitro with purified enzymes. Unlike AAV, Ad relies primarily on virally encoded enzymes for DNA replication (2). Like SV40 (29), AAV relies on a virally encoded, origin-binding DNA helicase (Rep), but AAV also appears to require a cellular helicase for DNA replication. We believe that this cellular helicase is the MCM complex. However, our affinity-purified MCM preparations contained various amounts of other proteins that are known to associate with MCM2 to -7 in vivo (see below). Presumably, we had several different complexes containing MCM, and we do not know which of these is the active species or, indeed, if one of the associated proteins is the active species.

In addition to AAV, the MCM complex has also been implicated in the replication of latent herpesvirus genomes. Both ORC and MCM have been shown to associate with the latent origins of DNA replication for Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus in vivo (8, 10, 24), and this is necessary for viral replication.

The MCM2 to -7 complex is assembled on eukaryotic chromosomes in the presence of the origin recognition complex by CDC6 and Cdt1 during the G_1 phase of the cell cycle (26). The complex is then activated during S phase by the addition of MCM10, CDC45, and the GINS complex. This leads to initiation of DNA synthesis at eukaryotic origins of DNA replication. Several lines of evidence suggest that the MCM complex then moves with the DNA polymerase complex. It is believed to be the replicative DNA helicase which separates the DNA strands ahead of the replication fork during chromosome replication.

It is unlikely that the MCM complex is assembled in the same way on AAV DNA as it is on chromosomal DNA. First, AAV replicates only after the helper virus has induced S phase, which is presumably after MCM has been assembled on host chromosomes. Assembly of the MCM complex at a cellular origin also requires the origin recognition complex, which has not been implicated in AAV DNA replication. In addition, proteins implicated in MCM assembly, cdc6 and cdt1, were not detected at significant levels in our purified MCM preparations compared to the MCM proteins (Fig. 5A and B; also mass spectroscopy results). Thus, there may be an alternative way of assembling an active MCM complex on replicating DNA. Elsewhere, we will demonstrate that the AAV Rep protein can

interact with MCM. Whether this interaction is the key to assembling the complex on AAV DNA remains to be tested. In addition to MCM2 to -7, several other cellular proteins are believed to be part of the MCM complex that is active during S phase. These include MCM10, GINS, and Cdc45. Of these, Cdc45 and MCM10 were detected in our purified His-tagged MCM6 preparation by immunoblotting (Fig. 5B). However, the level of these proteins and the GINS complex was significantly lower than the levels of the other MCM2 to -7 proteins as determined by silver stain SDS-polyacrylamide gel electrophoresis (PAGE) analysis. Therefore, it is unclear if these accessory proteins are required for AAV DNA replication or whether only a subset of the MCM complexes in our preparation are active. Further studies with individually purified recombinant proteins will enable us to determine the importance of each of these proteins.

We presume (but have not formally proven) that MCM functions as the replicative fork helicase during AAV DNA replication. In vitro AAV DNA replication, therefore, could provide a unique tool for studying the helicase activity of MCM. It is not clear whether MCM moves ahead of the fork on a DNA template or whether it remains in one place and threads DNA through the complex. The latter mechanism would create single-stranded loops that are then the templates for DNA synthesis (reviewed in reference 26). Either mechanism could be used for AAV DNA replication. We previously demonstrated that the Rep protein binds to two sequences within the AAV terminal repeat, RBE and RBE′, and then unwinds the terminal resolution site by pulling the DNA toward itself using its DNA helicase activity (5, 6, 20). In principle, a Rep-MCM complex could bind to the 5′ hairpin after reinitiation (Fig. 1) and unwind the displaced strand from the template as a loop, while Pol δ-PCNA extends the 3′ primer on the template strand. Further work is necessary to resolve these possibilities.

We and others (18, 25, 32, 34) have suggested that ssDNA binding proteins added to crude extracts or expressed in vivo stimulate AAV DNA replication. The RPA, Ad DBP, and herpes simplex virus (HSV) UL29 proteins have all been implicated. There is also evidence that RPA, Ad DBP, and the HSV ICP8 proteins can directly interact with the Rep protein and stimulate Rep-mediated nicking of the AAV origin (25). Furthermore, in studies of herpesvirus helper functions, the HSV UL29 gene, which codes for the herpesvirus ssDNA binding protein, was found to be an essential helper gene (34), along with the herpes helicase primase complex (UL5, UL8, and UL52). When expressed in human cells, UL29 and the helicase primase complex alone supported less than 1% of normal AAV replication levels seen in vivo in the presence of a wild-type herpesvirus infection, but their deletion reduced AAV DNA replication by 2 to 3 logs in the context of a herpesvirus infection (22, 34). Thus, unlike the case with Ad coinfection, expression of the herpesvirus ssDNA binding protein seemed to be essential. Finally, several groups have shown that ssDNA binding proteins and the Rep protein colocalize in vivo and that this depends on the presence of a functional AAV TR or replicating AAV DNA (12, 22, 25, 35). In light of this, it was surprising that we saw no requirement for ssDNA binding proteins in our reconstituted system. We suggest that the function of the ssDNA binding protein in vivo is likely to stabilize and protect the single-stranded replication products from nucleases or to facilitate DNA packaging. In our purified reconstituted system, however, where neither of these activities, endonucleases or packaging, is present, we have seen only a modest stimulation of DNA synthesis by RPA or Ad DBP (17). A similar modest in vitro stimulation has been seen by others (25, 32, 33). It is also likely that since the primary product of AAV DNA replication is ssDNA, AAV replication centers in vivo would attract ssDNA binding proteins.

All parvoviruses replicate their DNA by leading-strand displacement synthesis using a virally coded nonstructural protein (NS1) with properties similar to those of the AAV Rep protein (3). Autonomous parvoviruses remain quiescent until the cell enters S phase, while nonautonomous parvoviruses like AAV rely on a helper virus to induce S phase and prevent entry into mitosis. In both cases the virus waits for cellular replication enzymes to become available. There is evidence that the mouse autonomous parvovirus MVM also uses Pol δ, RFC, PCNA, and RPA for at least one step of its DNA replication (9), initiation from an internal 3′ dimer origin. In light of the common dependence on S phase, it is likely that the set of enzymes identified for AAV is also the one used by all other parvoviruses.

The fact that AAV uses the highly conserved enzymes Pol δ, RFC, PCNA, and MCM for DNA replication suggests an explanation for the remarkable promiscuity of AAV DNA replication. AAV can replicate efficiently not only in human cells but also in insect cells (14, 28). Moreover, parvoviruses populate both the vertebrate and invertebrate species. Thus, parvoviruses appear to have devised a method of DNA replication that uses a subset of some of the most highly conserved eukaryotic DNA replication enzymes.

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N.M. is an inventor of patents related to recombinant AAV technology and owns equity in a gene therapy company that is commercializing AAV for gene therapy applications.

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