JOURNAL OF VIROLOGY, Sept. 1994, p. 6029–6037
0022-538X/94/$04.00+0

Vol. 68, No. 9

Adeno-Associated Virus DNA Replication In Vitro: Activation by a Maltose Binding Protein/Rep 68 Fusion Protein

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Received 15 February 1994/Accepted 9 June 1994

The adeno-associated virus (AAV) nonstructural protein Rep 68 is required for viral DNA replication. An in vitro assay has been developed in which addition of Rep 68 to an extract from uninfected HeLa cells supports AAV DNA replication. In this paper, we report characterization of the replication process when a fusion of the maltose binding protein and Rep 68, expressed in *Escherichia coli*, was used in the assay. Replication was observed when the template was either linear double-stranded AAV DNA or a plasmid construct containing intact AAV DNA. When the recombinant plasmid construct was used as the template, there was replication of pBR322 DNA as well as the AAV DNA; however, linear pBR322 DNA was not replicated. When the plasmid construct was the template, replication appeared to initiate on the intact plasmid and led to separation of the AAV sequences from those of the vector, a process which has been termed rescue. There was no evidence that replication could initiate on the products of rescue. Rep 68 can make a site-specific nick 124 nucleotides from the 3′ end of AAV DNA; the site of the nick has been called the terminal resolution site. Our data are most consistent with initiation occurring at the terminal resolution site and proceeding toward the 3′ terminus. When the template was the plasmid construct, either elongation continued past the junction into pBR322 sequences or the newly synthesized sequence hairpinned, switched template strands, and replicated the AAV DNA. Replication was linear for 4 h, during which time 70% of the maximal synthesis took place. An additional finding was that the Rep fusion could resolve AAV dimer length duplex intermediates into monomer duplexes without DNA synthesis.

The human parvovirus adeno-associated virus type 2 (AAV) is classified as a dependovirus because of the requirement for coinfection with helper virus (either adenovirus [Ad] or herpesvirus) for optimal replication in cell culture (1, 2, 4, 21). In the absence of helper virus coinfection, the AAV genome integrates into the cellular genome to establish a latent infection (1). In several lines of human cells, integration has been reported to be within a defined locus on chromosome 19q13.3-qter (15–17, 27). Helper virus infection of the latently infected cell leads to rescue and replication of the AAV genome, with virion production. We have reported an in vitro assay which appears to be a model for the rescue and replication of AAV. It has many of the parameters determined for in vivo AAV rescue and replication (10). Indeed, many of the parameters of in vivo replication were determined after transfection of Ad-infected human cells with plasmid constructs the same as or similar to those used in the in vitro assay (11, 25). In particular, the in vitro assay requires an extract from HeLa cells coinfected by AAV and Ad; extracts from uninfected cells or cells infected by Ad or AAV alone do not support replication.

The in vitro assay also has specific template requirements. The AAV genome is a linear, single-stranded DNA (4,680 bases) with an inverted terminal repeat (ITR) of 145 bases (20, 28). The terminal 125 bases are an overall palindrome interrupted by two smaller, internal 21-base palindromes, one on either side of the overall axis of symmetry. When the palindromic region of the itr is folded on itself to maximize potential base pairing, a T-shaped structure is formed which is thought to act as a primer for DNA synthesis. The in vitro assay requires the ITR to be intact for rescue to occur; deletion of the terminal 55 bases blocks rescue but does not inhibit DNA synthesis. Again, these results mimic what is seen in vivo (25, 26).

The left half of the AAV genome encodes four regulatory proteins known as the Rep proteins (22). The two larger Rep proteins, Rep 68 and Rep 78, are required for DNA replication (10, 29). A putative intermediate in AAV DNA replication is a linear, duplex molecule cross-linked at one end by the hairpin structure. Rep 68(78) has been demonstrated to be able to nick such a structure at the terminal resolution site (TRS) between bases 124 and 125, creating a 5′ overhang (the transferred hairpin) (14). Rep 68(78) has also been shown to function as a helicase and an ATPase (14). These properties allow synthesis of the parental strand from the 3′ OH at nucleotide (nt) 125 to nt 1, using the transferred hairpin sequences as a template.

The relative simplicity of the AAV genome suggests that, with the exception of Rep 68(78), proteins involved in DNA replication are of cellular origin. However, the situation is made more complex by the requirement of helper virus coinfection for optimal replication. A basic question is whether helper virus coinfection simply allows sufficient Rep 68(78) expression for optimal replication or whether additional factors are provided. We have demonstrated that addition of Rep 78 produced in a vaccinia virus/T7 expression system to an extract from uninfected HeLa cells allows replication similar to that seen in our in vitro assay, which uses extracts from AAV/Ad-coinfected cells (19).

The expression of a maltose binding protein (MBP)/Rep 68 fusion protein in *Escherichia coli* has made available large amounts of this fusion protein (Rep 68^{MBP}) for detailed study

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of an in vitro reaction (6). The initial results described by Chiorini et al. (6) demonstrated that, in addition to the hairpinned conformation of the ITR, the ITR also contains a specific binding sequence $(GCTC)_3$ for Rep 68(78). Binding was detected to a plasmid construct from which the terminal 55 bases of the ITR had been deleted and to constructs containing only the $(GCTC)_3$ motif. The binding sequence itself was sufficient for Rep 68(78) binding and was not facilitated by hairpin formation. The in vitro replication appeared the same as the reaction using extracts from coinfected cells which we described previously (11). Analysis of template requirements, described in this report, leads to a more refined notion of the nature of initiation of DNA replication in the in vitro assay.

MATERIALS AND METHODS

Preparation of cell extracts. Extracts from uninfected HeLa cells and from HeLa cells coinfected with both Ad and AAV were prepared as described previously (11, 30) as a modification of the procedure originally described by Wobbe et al. (31).

Plasmids. Plasmid pAV2 has been described (18). It consists of the entire genome of AAV2 inserted into a pBR derivative by means of *Bgl*II linkers.

In vitro DNA replication. The standard reaction mixture (15 μl) contained 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.7), 40 mM creatine phosphate (pH 7.7), 7 mM $MgCl_2$, 4 mM ATP, 200 μM each CTP, GTP, and UTP, 100 μM each dATP, dGTP, and dTTP, 10 μM dCTP, 10 μCi of [α-^{32}P]dCTP (5,000 Ci/μmol; Amersham), 2 mM dithiothreitol, 6 mM potassium glutamate, 2.0 μg of creatine phosphokinase, approximately 100 μg of HeLa cell extract protein, 0.3 μg of plasmid, and approximately 1.0 μg of Rep 68^{MBP} or a control MBP fusion protein. Reactions which were to be analyzed quantitatively contained 100 μM dCTP. The reaction mix was incubated at 34°C for at least 4 h. Reaction products were proteinase K digested, phenol-chloroform extracted, and dissolved in 2.5 mM Tris HCl (pH 7.5)–0.25 mM EDTA. Products were analyzed by electrophoresis on 0.8% agarose gels. Gels were dried and exposed to Kodak X-AR film. PhosphorImager (Molecular Dynamics) scanning of dried gels was performed with ImageQuant version 3.0 software.

*Dpn*I digests were performed in 0.210 M NaCl (31) for 3 h and completion was assessed by ethidium bromide staining of gels prior to drying.

Dimer resolution. AAV head-head dimers were isolated from a reaction using plasmid pAV2 and an extract from Ad/AAV-coinfected cells. This reaction was not supplemented by Rep 68^{MBP}. Products were *Bam*HI digested and electrophoresed on an agarose gel, and dimers were recovered by QIAEX gel extraction. Dimer resolution was performed in the same reaction mix as was replication except that the reaction mix contained no HeLa cell extract and no [α-^{32}P]dCTP. One resolution reaction mix contained LacZ^{MBP}, and the other contained Rep 68^{MBP}.

AAV Rep 68^{MBP} protein. Construction, purification, and characterization of the protein have been described elsewhere (6). Briefly AAV sequences 327 to 1882 were cloned into pBR997 (New England Biolabs), an *E. coli* expression vector. The construct expressed a protein which contains the AAV Rep 68 protein (minus the 3 N-terminal amino acids and the 15 C-terminal amino acids and containing a 50-amino-acid LacZ fusion at the C-terminal) fused to a bacterial MBP. The fusion protein was isolated as recommended by New England Biolabs.

RESULTS

The requirement of Rep 68 or Rep 78 for initiation of replication at the AAV origin of replication, ITR, has been established in vivo and in vitro (10, 19, 23, 29). Rep 68^{MBP} has been shown to stimulate incorporation of labeling in vitro in an ITR-dependent manner (6). Similar results were obtained with Rep 78 produced from a recombinant vaccinia virus/T7 RNA polymerase system (19). In this report, the initiation of replication has been analyzed. The role of Rep in the rescue event is addressed as well.

Rep 68^{MBP}-supported replication. The ability of Rep 68^{MBP} to support AAV DNA replication is shown in Fig. 1A. A reaction using an unsupplemented extract from AAV/Ad-infected HeLa cells is compared with one using an extract from uninfected HeLa cells which was supplemented with Rep 68^{MBP}. The labeled species most comparable to earlier studies are the linearized plasmid (just below 9,416 bp) and the rescued product doublet (linear duplex AAV DNA and a linear duplex derivative pBR322 [hereafter referred to as pBR] DNA, above and below 4,316 bp). The latter have been characterized previously as containing full-length newly synthesized AAV strands (11, 12). These species appear roughly comparable, although there is more label in the rescue products in the Rep 68^{MBP}-supplemented reaction. Both of the rescued products in the Rep 68^{MBP}-supplemented reaction as well as in the unsupplemented reaction using extracts from AAV/Ad-infected cells are resistant to digestion with *Dpn*I, indicating that they contain at least one complete newly synthesized strand. In previously reported experiments, replication of pBR sequences in the rescued species had not been noted. Possible reasons for the observed results are listed in Discussion. Three reactions, each using an extract from uninfected HeLa cells which has been supplemented with a different MBP fusion protein, are shown in Fig. 1B. MBP has been fused to (i) a wild-type Rep 68 sequence (minus the 3-N terminal and 15 C-terminal amino acids), (ii) a mutant Rep 68 sequence (altered also in the putative nucleoside triphosphate [NTP] binding site), and (iii) LacZ. Use of a fusion protein containing a Rep with a missense mutation at amino acid 340 (Rep 68ntp^{MBP}) (5) severely reduces the extent of incorporation of radiolabeled dNTPs. Amino acid 340 is in the putative ATP binding site of Rep 68. Similarly, a LacZ^{MBP} fusion protein is inactive and serves as a negative control against which Rep 68ntp^{MBP} can be compared. Although the mutant protein does bind the ITR, the inability of Rep 68ntp^{MBP} to support DNA replication was anticipated because of the failure of this mutant to support replication in vivo (5). Without the addition of a fusion protein containing functional Rep 68, the uninfected HeLa cell extract supports virtually no incorporation of radiolabeled dNTPs.

Required reaction components. The requirements for in vitro replication were assessed (Table 1). Replication required HeLa cell extract, Rep 68^{MBP}, and exogenous DNA. The latter requirement was a clear indication that the replication observed was not extension of endogenous replication complexes. Efficient replication required Mg^{2+}, ATP, and an ATP-regenerating system. The extent of replication observed in the absence of added dNTPs suggests the presence of endogenous dNTPs. From calculation of the radioactivity incorporated by 18 h, the equivalent of about 75% of one new strand has been synthesized for every duplex AAV genome equivalent present in the original reaction mixture. In the reaction, there were about 100 molecules of Rep 68^{MBP} for every ITR present. Use of lesser concentrations of Rep 68^{MBP} resulted in proportional

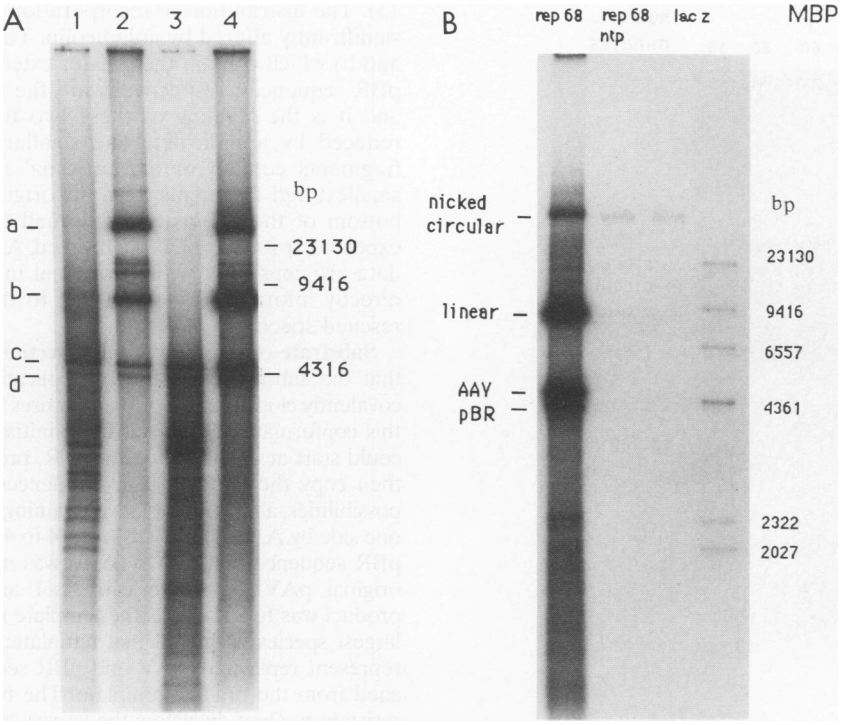

FIG. 1. (A) Comparison of replication products from two in vitro reactions, the first using an extract made from Ad/AAV-coninfected HeLa cells the second using an extract made from uninfected HeLa cells supplemented with Rep 68^{MBP}. Reactions were performed and analyzed as described in Materials and Methods; the substrate was pAV2. Lane 2 shows the results of a replication reaction using extracts made from cells coinfected with Ad and AAV; lane 1 shows a *Dpn*I digest of the products of this reaction; lane 4 shows the results of a replication reaction using an extract from uninfected HeLa cells with the addition of Rep 68^{MBP}; lane 3 shows a *Dpn*I digest of the products of this reaction. a, nicked circular plasmid; b, linear plasmid; c, rescued duplex AAV; d, rescued duplex pBR. (B) Comparison of replication using several MBP fusion proteins. To demonstrate the requirement for wild-type Rep 68^{MBP} fusion protein to induce replication of pAV2, three in vitro replication reactions were performed. The reactions were supplemented with different fusion proteins, Rep 68^{MBP}, Rep68ntp^{MBP} (which contains one mutant amino acid at position 340 of Rep 68), and LacZ^{MBP} (a fusion of LacZ sequences and MBP).

decreases in synthesis, while greater concentrations gave a slightly greater extent of synthesis (Table 2).

Time course of the reaction. Incorporation of label was linear for at least 4 h, with little or no lag (Fig. 2). The amount of label incorporated at 4 h was 70% of that seen at 18 h. The distribution of label changed significantly between 30 min and 4 h. At 30 min, much more of the label is in species migrating more slowly than the linearized plasmid or than the rescue products (AAV or pBR); the reverse is seen at 4 h (Fig. 3). There are no labeled species migrating between linearized plasmid and the rescue products. (If the rescued products did initiate replication, species intermediate between monomers and dimers would be expected). The ratio of rescued, replicated AAV to rescued, replicated pBR does not increase during the course of the reaction. (If the rescued products served to initiate replication, we would expect that the ratio of rescued, replicated AAV to rescued, replicated pBR would increase with time since only the rescued AAV form contains the TRS presumed necessary for the initiation of Rep-dependent replication.) These results are most consistent with the possibility that replication initiates while the AAV is still integrated and that rescued AAV and pBR do not serve as templates for initiation of further replication in this assay.

Initiation site. Hong et al. (11) showed that at least part of the ITR is required for replication because a deletion of the entire terminal palindrome inhibited replication. These data are consistent with in vivo evidence that replication initiates at the ends of the AAV genome (9). The experiment shown in Fig. 4 supports this conclusion. The replication reaction was carried out in the presence of aphidicolin to inhibit chain elongation by DNA polymerases alpha, delta, and epsilon (7,

TABLE 1. Requirements for in vitro replication

Component omitted	Relative activity
None (complete)	1.00
$MgCl_2$	0.104
ATP	0.168
CTP, GTP, UTP	0.770
dATP, dGTP, dTTP	0.378
Creatine phosphokinase, creatine phosphate	0.119
DNA	0.087
HeLa cell extract	0.083
Rep 68^{MBP}	0.086

TABLE 2. Titration of Rep 68^{MBP}

Concn of Rep 68^{MBP} relative to standard reaction conditions	Relative incorporation
0.5	0.42
1.0	1.00
1.5	1.31
2.0	1.08

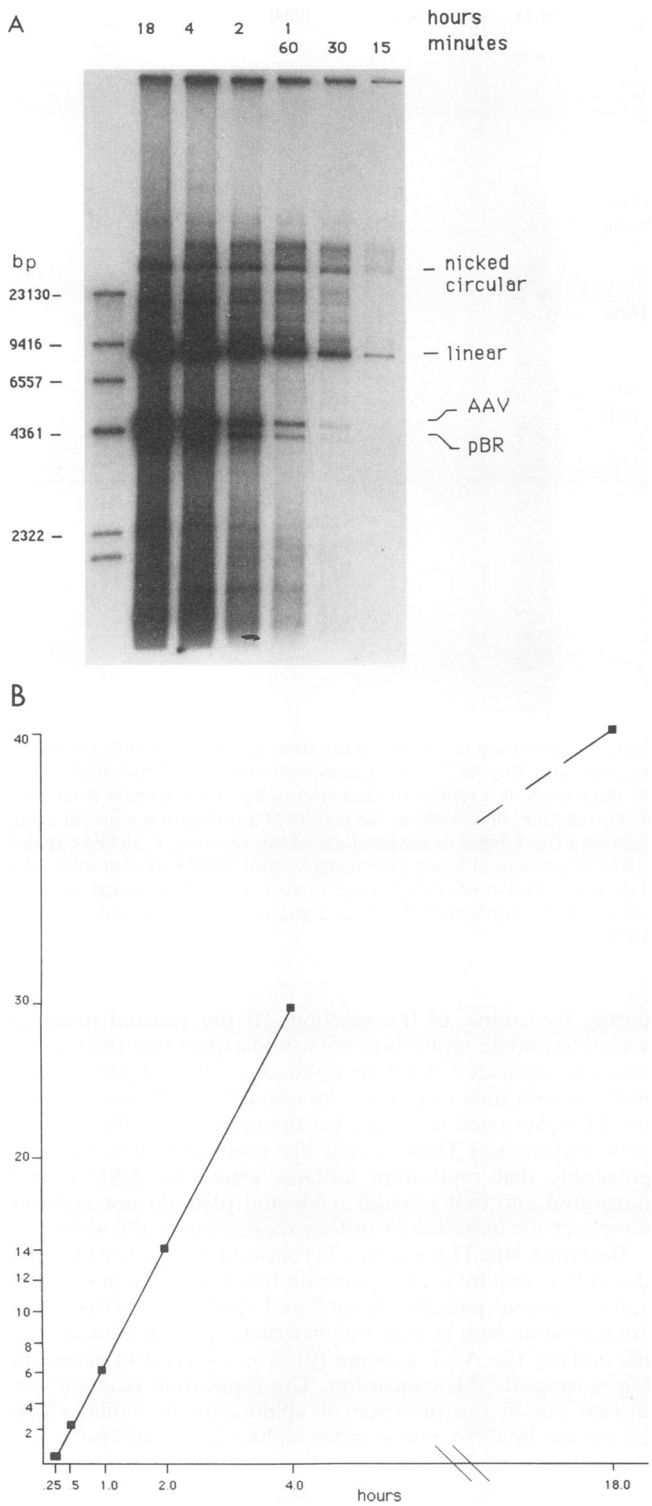

FIG. 2. (A) Time course of an in vitro replication reaction using an extract from uninfected HeLa cells supplemented with Rep 68^{MBP}. At the time points shown, an aliquot was removed and replication was stopped by adding EDTA to 7 mM and sodium dodecyl sulfate to 0.7%. Reaction products were then processed as described in Materials and Methods. (B) The rate of incorporation is linear. The graph shows the amount of incorporation at various time points of [α-^{32}P]dCTP into all species. The horizontal axis shows time after the start of the reaction. The vertical axis shows incorporation in PhosphorImager units.

13). The distribution of incorporation after *Bgl*I digestion was significantly altered by aphidicolin. The two *Bgl*I fragments (a and b) which contain the greater extent of both the AAV and pBR sequences, respectively, are the farthest from the ITRs, and it is the labeling of these two fragments which is most reduced by aphidicolin. The smaller, more heavily labeled fragments contain or are proximal to the ITRs. (The two smallest *Bgl*I fragments from the original construct are off the bottom of the gel, as are the small terminal *Bgl*I fragments expected from the ends of rescued AAV DNA.) Thus, these data are consistent with a terminal initiation site but are not directly informative with respect to the termini of the AAV rescued species.

Substrate conformation and direction of replication. The fact that the substrate used in the replication assay is ordinarily a covalently closed circular plasmid raises two considerations. First, this conformation might facilitate initiation. Second, replication could start at a nick within the ITR, proceed through pBR, and then copy the bulk of AAV sequences. To test both of these possibilities, a linear template containing the right ITR flanked on one side by AAV sequences (nt 814 to 4680) and on the other by pBR sequences (nt 375 to 3539) was made by digestion of the original pAV2 substrate with *Ase*I and *Sac*I. This digestion product was functional as the template in the assay (Fig. 5). The largest species is the original template; the two smaller species represent replicated AAV and pBR sequences which were rescued from the original template. The two rescued species were resistant to *Dpn*I digestion; the largest species was not. There is a minor band which is present in the *Dpn*I-digested lane and which is labeled a in Fig. 5. Most, if not all, of the label in the linear band consists of molecules in which pBR has been replicated but AAV sequences have not. Upon *Dpn*I digestion, this material is digested to a pBR-size fragment. Band a represents a fraction of this replicated pBR which failed to digest at the AAV *Dpn*I site that is closest to the ITR. Although there are 30 *Dpn*I sites in the plasmid, by a statistical anomaly there is a segment of AAV of approximately 2,000 bases which is free of *Dpn*I sites and which is separated from pBR by only the one site mentioned above. (The two *Dpn*I sites flanking this segment are shown in Fig. 5.) Replication of pBR which remains attached to unreplicated AAV followed by occasional (25%) failure to digest at this one *Dpn*I site results in this band, which is approximately 2,000 bases longer than the pBR fragment.

This assay makes several points: (i) the template can be a linear molecule, (ii) replication initiating at the ITR can go in either direction, and (iii) because pBR sequences were replicated, initiation of replication in the pBR direction must have occurred before rescue. The rescued pBR fragment contains no TRS which is presumed necessary for Rep 68-initiated replication.

Necessity of ITR for Rep 68^{MBP} replication. The role of the ITR in replication of AAV and pBR sequences is illustrated in Fig. 6. pAV2 was digested with *Bgl*II, which cleaves in the linker between the AAV ITR and pBR. The products are full-length linear AAV DNA, which is the equivalent of linear duplex AAV DNA and full-length linear pBR DNA. When the *Bgl*II cleavage products were used as templates in the in vitro assay, only the AAV fragment which contained the ITR was replicated. These results are consistent with the notion that initiation occurs only within the ITR and that when the ITR is a junction between AAV and pBR, either AAV or pBR may be replicated.

A dimeric replicative intermediate. Linear, duplex AAV dimers have been observed as replicative intermediates in vivo and in vitro with the use of extracts from AAV/Ad-infected cells. The linear, duplex dimers produced in vitro have been

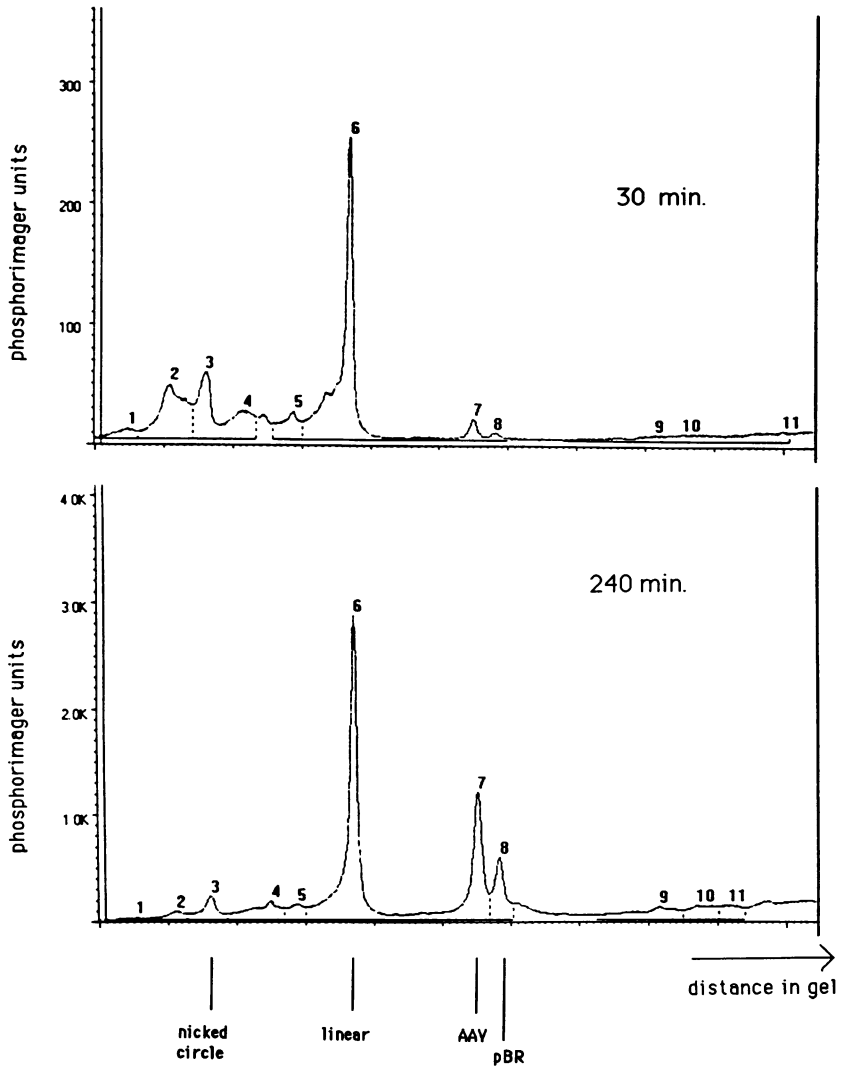

FIG. 3. PhosphorImager tracing of the gel whose autoradiogram is shown in Fig. 2A. To assess more quantitatively the relative amount of labeled DNA in each band, a PhosphorImager analysis was performed on each lane of the gel of Fig. 2A. Shown are tracings of the 30- and 240-min lanes, with the DNA species represented by several peaks indicated at the bottom. The area under each peak is proportional to the radioactivity in that band on the gel. The horizontal axis represents distance on the gel, measured from the well. The vertical axis indicate PhosphorImager units showing relative radioactivity.

characterized recently as head-to-head and tail-to-tail tandems of the type predicted by the current model for AAV DNA replication (12). However, as illustrated in Fig. 1b, dimers (migrating at the position of linearized plasmid) which should be resistant to *Dpn*I are not observed (23). This could reflect the possibility that dimers are not formed with Rep 68^{MBP} or that at the concentration used, Rep 68^{MBP} very quickly resolves any dimeric structure. That the latter can occur is suggested by the data illustrated in Fig. 7. An in vitro replication assay was carried out with an unsupplemented AAV/Ad-infected cell extract. The DNA was digested with *Bam*HI (which digests AAV at nt 1045), and the fragments were separated by gel electrophoresis. A fragment of approximately 2,000 bases in length which represents AAV head-head dimers was gel recovered. The gel-recovered DNA was further incubated in buffer at 34°C in the presence or absence of Rep 68^{MBP} (no cell extract was present). The negative control contained $LacZ^{MBP}$ instead of Rep 68^{MBP} and should have also contained any possibly contaminating nucleases. The amount of head-to-head dimers was greatly reduced by exposure to Rep 68^{MBP}, as opposed to exposure to $LacZ^{MBP}$. Table 3 gives relative values for the radioactivity in the dimer and hairpin bands and their ratios as determined by PhosphorImager analysis. The assay might also have been expected to cut the TRS in the extended termini, leading to loss of a short single-stranded piece on the 5′ side of the nick (due to helicase) and subsequent hairpinning of the overhang. An extended terminus fragment would then migrate as a hairpinned end. Thus, the data do not argue against AAV dimers as replicative intermediates in this reaction.

DISCUSSION

The ready availability of Rep 68^{MBP} has made a more detailed analysis of the in vitro assay of AAV DNA replication possible. There are three conclusions from the experiments

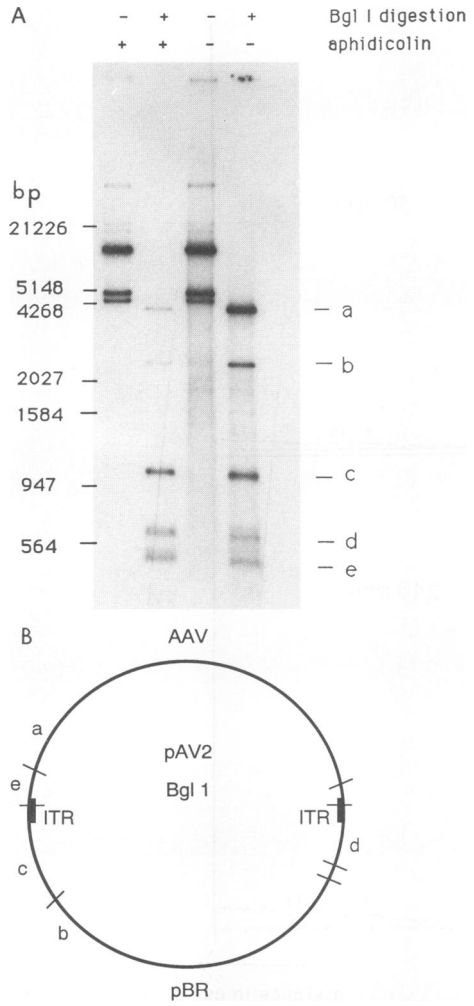

FIG. 4. (A) Replication of pAV2 induced by Rep 68^{MBP} initiates at or near the AAV ITR. Two reactions were performed as described in Materials and Methods except that one reaction was performed in the presence of 20 μM aphidicolin. Shown is an autoradiogram of the products of these reactions either undigested or after *Bgl*I digestion. In the presence of aphidicolin, there is preferential labeling of restriction fragments which were derived from regions of the plasmid which included or were adjacent to the AAV ITRs. a through e are *Bgl*I restriction fragments. (B) *Bgl*I restriction map of pAV2.

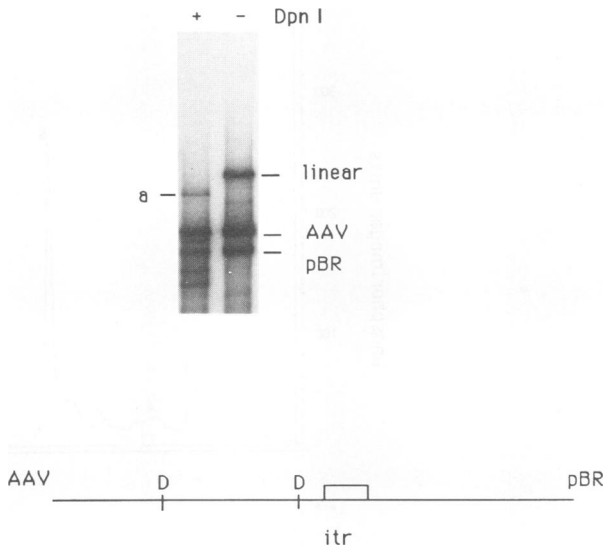

FIG. 5. Replication can proceed in either direction from the AAV ITR. Shown are the products of a replication performed as described in Materials and Methods except that the substrate in the reaction was the large *Ase*I-*Sac*I fragment of pAV2. As shown, this is a linear piece of DNA with approximately 4,000 bases of AAV sequence on one side of the AAV ITR and 3,200 bases of pBR sequence on the other side. Also shown is a *Dpn*I digest of this material. The derivation of the band designated a is described in the text. D's indicate the locations of two *Dpn*I sites in this construct.

reported in this paper. (i) The results are in accord with all of the parameters of DNA replication observed previously in vivo and in vitro (with the exception of pBR replication, discussed below). (ii) A single point of initiation at the TRS is consistent with the observed results with both wild-type constructs and the previously observed results with the mutant from which the terminal 55 nt had been deleted at both ends of the AAV insert (11, 25, 26). (iii) Rep 68^{MBP} can resolve linear, duplex dimers without replication.

The results of the in vitro assay using an extract from uninfected HeLa cells supplemented with Rep 68^{MBP} are similar to both the results of an in vitro assay using an extract from AAV/Ad-infected cells and the results observed in vivo after either transfection or infection. A significant feature of both in vitro assays is the use of an infectious clone of AAV DNA in a pBR vector as a template. Use of this template with Rep 68^{MBP} leads not only to rescue of the AAV insert but also to replication of both AAV and pBR DNAs. This was not apparent in the original characterization of the in vitro assay using AAV/Ad extracts but has now been observed. We believe that the failure to observe pBR replication by Hong et al. (11) reflected overly stringent conditions of *Dpn*I digestion, so that only fully unmethylated (both strands newly synthesized) DNA was not digested. The failure to observe pBR replication in vivo could represent the inability of free pBR DNA to reinitiate replication because of lack of a TRS, whereas the AAV DNA is greatly amplified. Our current model can now account for earlier in vitro replication studies using an AAV template with the terminal 55 nt deleted, which has been confirmed with Rep 68^{MBP} (data not shown). Deletion of 55 bases from the ends of the ITRs of the AAV insert prevents rescue from the vector but does not block DNA replication of the intact plasmid. Most models of AAV replication hypothesize the use of a hairpin primer for initiation. Originally, we suggested that the ability of the deletion mutant to be replicated implied a second form of initiation, since a hairpin structure could not be formed. Below we describe alternative models for initiation of replication which can account for initiation both in the plasmid containing intact AAV as well as in the plasmid containing the 55-base deletion.

Two models which could account for the rescue and replication of AAV DNA in the in vitro assay described in this report are shown in Fig. 8. The key point is that replication of pBR must initiate while AAV and pBR are still joined. If AAV replication were to initiate after AAV and pBR have become separated, the ratio of AAV replication to pBR replication would increase with time. This is not the case. Therefore, AAV replication as well as pBR replication must initiate before AAV is rescued from the vector. This idea is in accord with the absence of replicative intermediates migrating at positions between those of linearized plasmid and rescue products. In

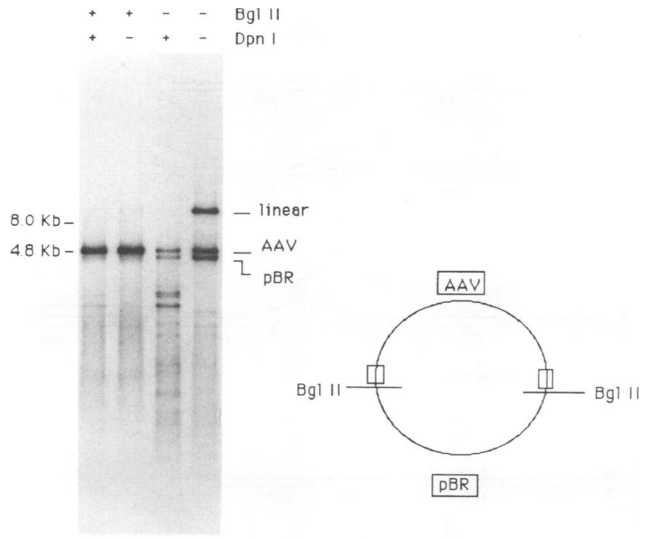

FIG. 6. Replication of linear duplex AAV DNA. Two replication reactions were performed as described in Materials and Methods. The substrate of one reaction was intact pAV2. The substrate of the other reaction was pAV2 which had been digested into two restriction fragments with *Bgl*II. *Bgl*II digests in the linker adjacent to AAV as shown in the diagram, resulting in a fragment which contains the entire AAV genome with a few bases of *Bgl*II linker attached to the outside of each AAV ITR and a pBR fragment which also has a fragment of *Bgl*II linker at each end. After the replication reaction, products were separated on an 0.8% agarose gel either with or without prior *Dpn*I digestion. In the reaction which uses *Bgl*II-digested pAV2 as a substrate, duplex AAV replicates, while the pBR fragment, which has a slightly greater mobility on the gel than AAV, does not replicate.

TABLE 3. Dimer resolution by Rep 68^{MBP}

Band	Relative radioactivity		
	Rep 68^{MBP}	$LacZ^{MBP}$	Untreated
Head-head dimer	0.175	2.825	4.291
Hairpin	0.659	0.424	0.110
Ratio, head-head dimer/hairpin	0.27	11.8	39.0

the first model, Rep nicks at the TRS in the ITR and the 3′ OH produced serves as a primer. Synthesis starts and proceeds toward the junction of the AAV ITR with pBR; just before the junction with pBR, the newly synthesized strand folds back on itself and continues then to synthesize the rest of the AAV genome. This is simply a slight variation on the current model for initiation of AAV DNA synthesis on duplex replicative intermediates and is consistent with the model of AAV DNA replication proceeding by a single-strand displacement mechanism. If the newly initiated strand does not fold back on itself, it continues synthesis through the pBR sequences. One of the species produced by *Dpn*I digestion would correspond to this type of synthesis (Fig. 1A). Constructs in which the terminal 55 nt have been deleted from both ITRs cannot be rescued in vivo or in vitro; the whole plasmid is replicated (26). The latter alternative (no foldback) of the first model would account for this observation.

In the second model, Rep also nicks at the TRS in the ITR and the 3′ OH produced serves as a primer. Synthesis starts and proceeds toward the junction of the AAV ITR with pBR. Then concurrently with the nick at the TRS site, Rep or a

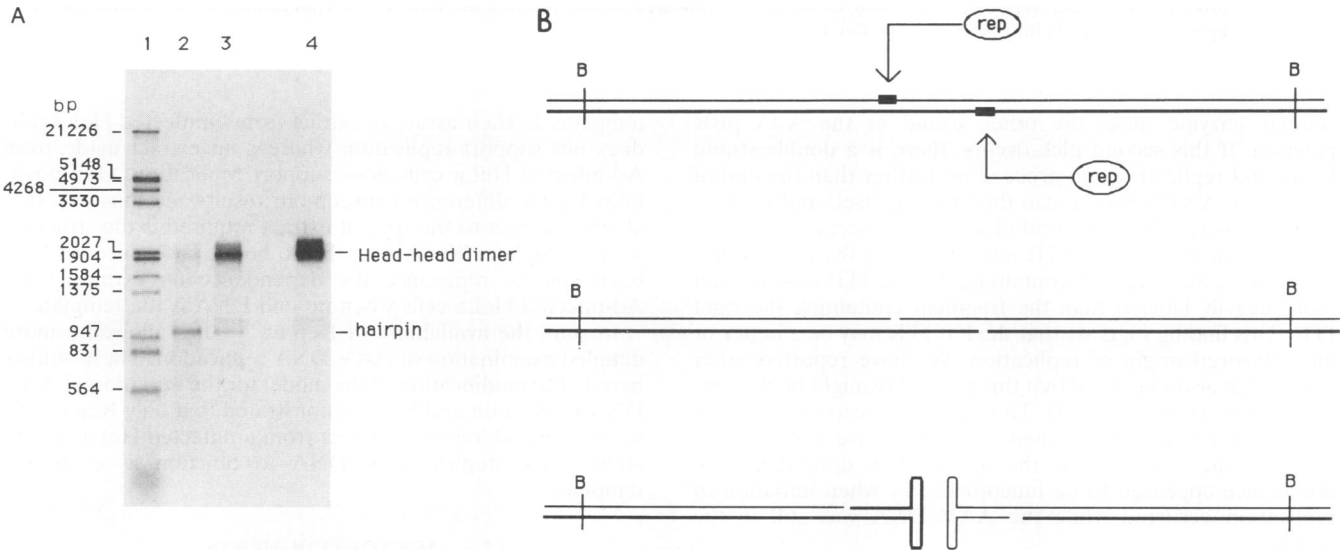

FIG. 7. Resolution of a head-to-head dimer. A replication reaction was performed with an extract made from HeLa cells coinfected with AAV and Ad. The radioactively labeled replication products of this reaction were proteinase K digested, phenol-chloroform extracted, and alcohol precipitated. Prior to separation on an 0.8% agarose gel, the replication products were digested with *Bam*HI, an enzyme which cuts at base 1045 in AAV. After *Bam*HI digestion AAV head-to-head dimer fragments will appear as a band of about 2,000 bases. This band was gel recovered. Subsequently, this DNA was incubated with either Rep 68^{MBP} or $LacZ^{MBP}$ in the absence of any cellular extract as described in Materials and Methods. The products of this incubation were separated as shown. Lane 1 shows incubation with Rep 68^{MBP}, lane 2 shows incubation with $LacZ^{MBP}$, and lane 3 shows the gel-recovered material without any subsequent incubation. After incubation with Rep 68^{MBP}, there was a relative decrease of head-to-head dimer and a relative increase of DNA at the position of a single rescued AAV hairpin fragment compared with incubation with $LacZ^{MBP}$. (B) Diagram illustrating the model for the resolution reaction of panel A. Rep 68^{MBP} nicks each strand at its terminal resolution site. After separation, through either helicase activity or branch migration of the DNA sequence between the two nicking sites, two duplex DNA molecules have been produced, each with a terminal hairpin and each of the size expected for a single rescued AAV fragment. B, *Bam*HI site; ▬, TRS.

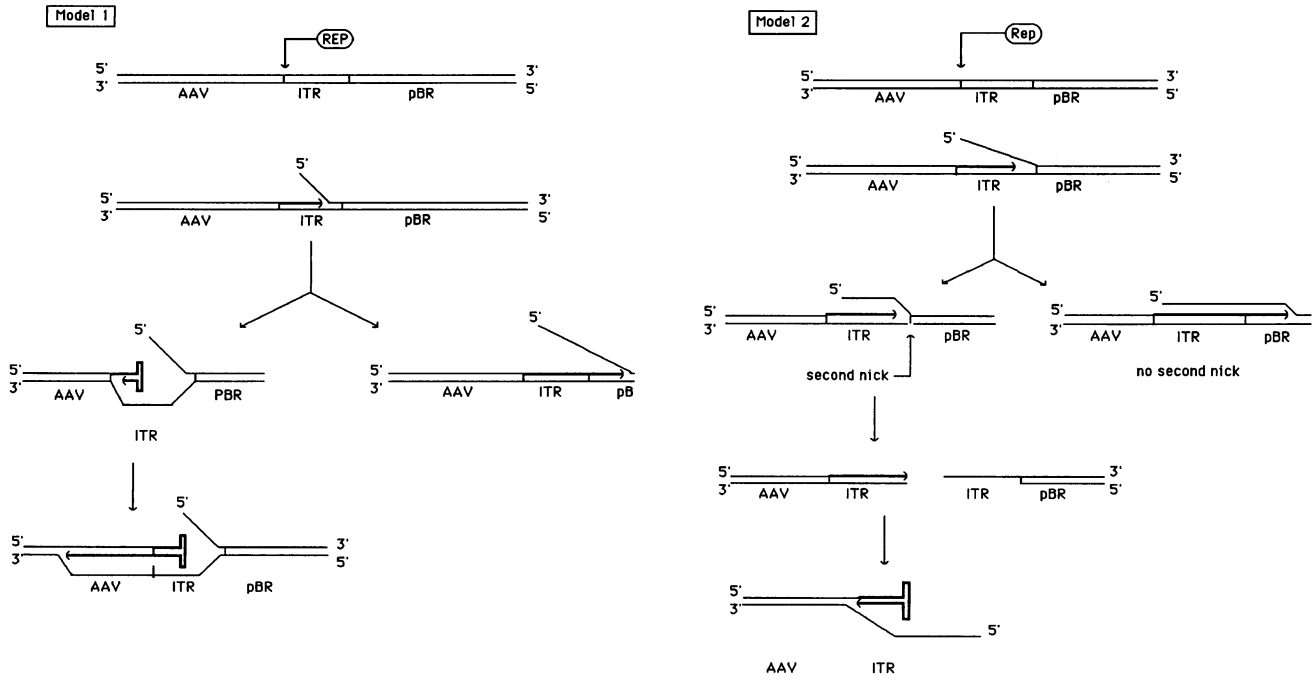

FIG. 8. Two models for initiation of replication in pAV2 as induced by Rep 68^{MBP}. In model 1, Rep 68^{MBP} nicks the DNA at the TRS found on the inboard side of an ITR. Replication initiates at this nick and proceeds in the 5'-to-3' direction, i.e., through the terminal repeat. As the replication complex passes through the terminal repeat, the replicating strand may make the transition to the hairpin conformation. The replicating strand will no longer be base paired to the original template strand but is in the configuration to base pair with itself. The replicating strand can then serve as its own template. After such a template strand switch, replication will of necessity proceed back into AAV sequences. The consequence of this mode of replication initiation is that one terminus of a duplex AAV molecule is released from the plasmid. If the replicating strand fails to hairpin, replication will proceed into pBR. In model 2, nicking and initiation of replication are the same as for model 1. In this model, however, a second nick may occur at the AAV-pBR junction. The consequence is that AAV replication cannot extend past the terminal base of AAV. The bulk of AAV is then replicated by folding of the newly replicated strand, forming the standard hairpin primer. If the second nick does not occur, replication proceeds into pBR as in model 1.

cellular enzyme nicks the other strand at the AAV-pBR junction. If this second nick occurs, there is a double-strand break and replication will proceed no further than the end of AAV. The AAV terminus can then fold on itself and serve as a hairpin primer. If the second nick does not occur, replication will proceed through the ITR into pBR as in the first model.

In Fig. 4, the fragment containing the left ITR is somewhat more heavily labeled than the fragment containing the right ITR. This finding suggests that the left ITR may be a better or more favored origin of replication. We have reported other results that also suggested that this possibility might be the case in vivo after transfection (3). The earlier suggestion was based on the observation that when the ITRs have different sequences, the sequence of the left ITR is dominant. The dominance appeared to be functional only when initiation of replication occurred while the AAV DNA was still in the vector.

The experiment shown in Fig. 7 illustrated that Rep 68^{MBP} was able to cleave AAV duplex dimers without the addition of cell extract. A head-to-head or tail-to-tail dimer would have TRSs on complementary strands separated by 124 nt; branch migration and/or Rep 68^{MBP} helicase activity could separate the two unit monomers. Branch migration could be facilitated by the two smaller palindromes on either side of the overall axis of symmetry.

Ni et al. (23) have recently published an alternative in vitro assay which uses no-end DNA (a duplex AAV genome which is covalently closed at each end by one copy of the ITR) as the template. In their assay, an extract from uninfected HeLa cells does not support replication whereas an extract made from Ad-infected HeLa cells does support replication. One possibility for the difference between our results and those of Ni et al. with respect to the type of extract required is the structure of the template. We suspect this to be the case since we have been able to reproduce the dependence on extracts from Ad-infected HeLa cells when no-end DNA is the template.

In sum, the availability of Rep 68^{MBP} has allowed a more detailed examination of AAV DNA replication. These studies have led to modification of the model for the initiation of AAV DNA replication and have demonstrated that only Rep 68^{MBP} needs to be added to an extract from uninfected HeLa cells to allow linear duplex AAV DNA to function as a specific template.

ACKNOWLEDGMENTS

We thank E. Falck-Pedersen, N. Hackett, M. O'Donnell, and R. M. Linden for helpful discussion and N. Cortez for technical assistance.

This research was supported by Public Health Service grant GM 50032.

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