In vitro replication of adeno-associated virus DNA

G. Hong, P. Ward, and K. I. Berns

Hearst Microbiology Research Center, Department of Microbiology, Cornell University Medical College and Program in Molecular Biology, Cornell University Graduate School of Medical Sciences, 1300 York Avenue, New York, NY 10021

Communicated by B. L. Horecker, February 1, 1992 (received for review November 12, 1991)

ABSTRACT An in vitro assay for adeno-associated virus (AAV) DNA replication has been developed. The substrate is a plasmid containing the duplex form of AAV DNA in pBR322. The AAV insert is excised or rescued from the plasmid by extracts of uninfected cells. Replication was assayed by production of full-length excised AAV DNA resistant to Dpn I digestion. The following results were obtained. (i) Only extracts of cells coinfected with AAV and adenovirus replicated the excised insert. (ii) Density label experiments showed semiconservative replication. (iii) Only the excised AAV insert was replicated; pBR322 sequences were not. (iv) Replication was dependent on the presence of the AAV terminal repeat. (v) If the terminal 55 bases were deleted from both ends of the AAV insert, no rescue took place; replication occurred and both AAV and pBR322 sequences were replicated. We conclude that the AAV terminal repeat is essential for DNA replication but that under some conditions an initiation mechanism that does not involve hairpin priming may be used.

The adeno-associated virus (AAV) genome has a palindromic inverted terminal repeat thought to serve as a “hairpin” primer to initiate DNA replication (1-3). In vivo AAV DNA replication appears to occur by a single-strand displacement mechanism leading to a duplex replicative intermediate covalently crosslinked at one end by the hairpin primer (Fig. 1) (2, 3). To resolve the hairpin, the AAV-encoded rep 68/78 protein specifically nicks the hairpin at a point on the parental strand directly opposite the original 3’ terminus (4). As a result of the nick, the 3’-terminal 125 bases are transferred from parental to progeny strand and are inserted in the process. The nick/transfer process leaves a 3’-OH at the end of the shortened parental strand, which then serves as a primer for synthesis to fill in the gapped region created by the transfer. In this reaction, the transferred hairpin sequence acts as the template.

AAV DNA replication can be initiated from two very different substrates. In a permissive cell, in the presence of helper virus, the starting point for a productive infection is a small, linear, single-stranded DNA whose palindromic 3’ terminus is free to form a hairpin and serve as a primer. Alternatively, in a latently infected cell the viral DNA is integrated as a duplex molecule into the host genome (5, 6). In the latter case, rescue or excision of the integrated genome is required for virion production. However, it is not known whether excision per se precedes the onset of free virion DNA synthesis or is a consequence of it. In vivo, the AAV rep gene is required in trans for rescue from the integrated state and for replication (7, 8).

A plasmid containing the duplex form of AAV DNA in pBR322 is fully infectious in adenovirus-infected HeLa cells (9, 10). In this case, the plasmid appears to be a model for the integrated form of the AAV genome. Transfection experiments have demonstrated that an intact terminal repeat is necessary for rescue of AAV from the plasmid and replica-

tion of the AAV sequences (11, 12). There is no evidence for replication of the vector sequences (9). However, if the terminal 55 bases are deleted from both ends of the AAV insert, there is no excision or rescue of the AAV sequences, but the entire plasmid appears to replicate (A. Beaton, G. Blumont, and K.I.B., unpublished data).

Recently, we reported an in vitro rescue of the duplex form of AAV DNA from a plasmid (13). The AAV construct used contained the simian virus 40 (SV40) regulatory region [including the SV40 origin (ori)] in place of the leftwardmost AAV promoter at map position 5. The presence of the SV40 ori allowed the construct to be replicated in a SV40 large tumor antigen (T antigen)-dependent manner. In this in vitro system, the AAV insert was rescued as a linear duplex molecule. Rescue or excision was enhanced by, but not absolutely dependent on, the addition of T antigen. The factors responsible for the actual excision were present in an extract from uninfected HeLa cells. The rescue products were linear duplex pBR and linear duplex AAV DNAs and differed from those reported by Gottlieb and Muzyccza (14), because the ends of both were covalently closed by the palindromic region (nucleotides 1-125) of the AAV terminal repeat.

Development of an in vitro assay for AAV DNA replication has been complicated by the fact that various DNA polymerases can use the hairpin primer, thus hindering evaluation of the physiologic significance of the assay. Im and Muzyczka (4) have made significant progress with a duplex AAV substrate in which both ends are covalently crosslinked by the terminal hairpin. In this paper, we describe an in vitro assay for AAV DNA replication by using a plasmid containing the duplex form of AAV DNA in pBR322 as a substrate. The in vitro results are in accord with the in vivo data, including the possibility that under some conditions initiation of AAV-specific DNA replication may involve a mechanism other than a hairpin primer.

MATERIALS AND METHODS

Cells. HeLa-S3 cells were grown as suspension cultures in Joklik’s minimal essential medium (JMEM) supplemented with 2.5% (vol/vol) fetal calf serum and 2.5% (vol/vol) calf serum. Human 293 cells were grown in monolayer cultures in Dulbecco’s minimal essential medium supplemented with 5% fetal calf serum and 5% calf serum.

Viruses. AAV type 2 was propagated by transfecting cells with pSM620 (9) and infecting the same cells with adenovirus type 2. Lysates were made by freezing and thawing infected cells, which were then heated to 56°C for 10 min and used as virus stocks. Virus titer was determined by the infectious center assay (15). Adenovirus type 2 was prepared and titrated with human 293 cells as described (16).

Plasmids. Plasmid pSM620 has been described (9). Plasmid pGM106 was created by insertion of the 4400-base-pair Bal I fragment of AAV into the Fst I site of pBR322 vector (14).

Abbreviations: AAV, adeno-associated virus; SV40, simian virus 40; T antigen, large tumor antigen.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
Fig. 1. Model of AAV DNA replication. ABCA'D represents the terminal repeat. B and C represent the small internal palindromes within the overall terminal palindrome ABCA'. Primed letters represent sequences complementary to unprimed letters.

Plasmid pLB2904 was created by insertion of the large Sma I fragment of pHM401 (7) into the EcoRV site of pAT153 (17), a high-copy variant of pBR322. pAT153 was found to be equivalent to pBR322 in in vitro DNA replication assays (18). Plasmids were propagated in Escherichia coli HB101. Plasmid DNAs were prepared by the alkaline extraction method (19). The plasmids are illustrated in Fig. 2.

Infection. HeLa cells (500 ml; 5 \times 10^5 cells per ml) in suspension culture were infected with adenovirus and/or AAV by removing the culture medium, washing the cells once with 40 ml of serum-free JMEM, and infecting the cells with 40 ml of serum-free JMEM containing virus at a multiplicity of infection of 5 plaque-forming units (pfu) per cell for adenovirus and 20 pfu per cell for AAV. The plates were incubated for 1 hr at 37°C and gently rocked every 10 min.

After incubation, the cells were transferred to a spinner flask and 500 ml of complete JMEM was added; cells were then incubated at 37°C in 5% CO2/95% air for 30 hr.

Preparation of HeLa Cell Extracts. Extracts were prepared essentially as described (20) and modified by Ward and Berns (13).

In Vitro DNA Synthesis. The AAV DNA replication reaction was carried out as described (13) using plasmid pSM620, pGM106, or pLB2904 in HeLa cell extracts made from mock-or virus-infected HeLa cells without SV40 T antigen. Dpn I (Boehringer Mannheim) digests were performed in 0.210 M NaCl for 36 hr (20). Initially, 24 units of Dpn I per \mu g of DNA was used; an equal amount of Dpn I was added at 12 hr. DNA products were electrophoresed through 0.8% agarose gels in 1 \times TBE buffer (90 mM Tris/64.6 mM boric acid/2.3 mM EDTA, pH 8.3); the gel was dried and autoradiographed at room temperature without an intensifying screen or at −80°C with one intensifying screen (DuPont Cronex Lightning Plus) and exposed to Kodak XAR film.

Southern Blotting. Southern hybridizations were performed by electrophoresing the unlabeled DNA replication products in agarose gels and blotting the agarose gel onto a nylon membrane (21). Probes were prepared by random oligonucleotide priming (22). Filters were prehybridized and hybridized at 67°C in 10× Denhardt’s solution (23)/6× SSCP (1× SSCP is 0.12 M NaCl/0.015 M sodium citrate/0.13 M KH2PO4/0.002 M EDTA, pH 8.0)/0.5% SDS/50 mM Tris-HCl, pH 7.5/100 \mu g of calf thymus DNA per ml. Prehybridizations were for 3 hr and hybridizations were for 24 hr. The filters were washed at 67°C in six successive buffers containing 1 mM EDTA. The first four buffers contained 6× SSC (1× SSC is 0.15 M NaCl/0.015 M trisodium citrate, pH 7) and 1% SDS, the fifth buffer contained 0.5× SSC, and the sixth buffer contained 0.2× SSC.

CsCl Equilibrium Gradient Centrifugation. DNA was synthesized in a reaction mixture containing 5-bromodeoxyuridine triphosphate in place of dTTP and DNA products were analyzed by equilibrium centrifugation in 4 ml of CsCl solution (0.1 M Tris-HCl, pH 8.0/0.01 M EDTA; density, 1.73 g/ml). Centrifugation was performed with an SW50.1 rotor in the Beckman L7-55 ultracentrifuge at 25°C at 55,000 rpm for 40 hr. Gradients were fractionated from the bottom of the centrifuge tube. The density of selected fractions was determined with a refractometer, and the DNA products in each CsCl fraction were monitored by electrophoresis through a 0.8% agarose gel.

RESULTS

The infectivity of plasmid constructs containing the intact duplex form of the AAV genome and the availability of the SV40 in vitro assay for DNA replication (18) led us to try to develop a comparable system for AAV DNA replication. The particular attraction of the SV40 system was that T antigen was the only viral protein required. All other components were present in a cytoplasmic extract of uninfected HeLa cells. As discussed in the Introduction, the use of the plasmid construct seemed likely to inhibit or prevent nonspecific priming by a 3' hairpin structure. The goal was to achieve synthesis of full-length AAV DNA in a system that required an extract from cells productively infected by AAV (with an adenovirus helper) and that required sequences of the AAV terminal repeat in cis for replication. Also, part of the goal was to achieve results in accord with in vivo experiments, to wit: replication of only the AAV sequences if the full terminal repeat was present but replication of the entire plasmid construct with no concomitant AAV insert rescue if the terminal 55 nucleotides were deleted from both ends of the AAV insert.

Requirement for AAV/Adenovirus CoInfection. To test whether DNA synthesis specific for AAV/adenovirus-
coinfected cells could be detected, cytoplasmic extracts were prepared from uninfected HeLa cells or from HeLa cells that had been infected with either AAV or adenovirus or both. The substrate was a plasmid that contained an intact copy of the duplex form of the AAV genome. All four extracts incorporated labeled dNTPs and all four produced a product comparable in length to the AAV excision product described previously (13). To determine which of the labeled species represented replication, DNA was digested with Dpn I, a restriction enzyme specific for methylated DNA. Newly synthesized DNA would be unmethylated in at least one strand and thus resistant to digestion. Only the extract of AAV/adenovirus-coinfected cells produced a species resistant to Dpn I digestion (Fig. 3, lanes 6 and 12). The Dpn I-resistant species migrated at a rate equivalent to the upper half of the band representing the excised species (lane 6). In experiments of this type, it was possible to sometimes discern two bands in the "rescued species" position when replication had occurred. The reaction was dependent on the addition of exogenous template (lanes 13 and 14). The reaction was lengthy (16 hr). The first Dpn I-resistant DNA was seen at ~4 hr and increased linearly to a maximum at 16 hr (data not shown). Repair-type synthesis reached a maximum by 4 hr. Equal amounts of DNA were used in all reactions and were applied to all the lanes. Frequently (see Figs. 3 and 6), but not always, more label was incorporated when the AAV/adenovirus extract was used.

**Density Labeled Replication Species.** To confirm that the Dpn I-resistant species produced by the AAV/adenovirus extract was a product of semiconservative replication, a replication reaction was carried out with BrdUTP used in place of TTP. In a CsCl density gradient, the excision species banded at both the light density and at a hybrid density. Species corresponding to forms II and III banded predominantly, if not completely, at the light density (Fig. 4). These data support the conclusions presented in Fig. 3 that true (semiconservative) replication occurred specifically in the excision species and that other radioactive DNA species were generated by nonspecific repair. It also was clear that only one round of replication had occurred, since there was no evidence for a heavy density product in which both strands contained BrdUrd in place of T. A small amount of form III DNA (~5%) banded at the hybrid density and there was no detectable hybrid density form II.

A similar density-label experiment was performed in the absence of radiolabel. The reaction products were detected by Southern blotting (data not shown). By PhosphorImager analysis of the distribution of the species detected by the AAV probe, we estimated that 10% of the rescue product was replicated. This represented 1% of the total input DNA. In Fig. 4, it appears that one-half of the label is at the hybrid density position. If we assume rough equivalence between the two experiments, we can estimate that on average only 5% or less of each strand had been replaced by repair-type synthesis in the rescue species at the light density position (0.1 × 0.5 = 0.05).

**Only the AAV Sequences Are Replicated.** Transfection of infectious clones under permissive conditions led to rescue and replication of the AAV sequences, but no evidence for vector sequence replication was observed (9). To learn which sequences were synthesized in the in vitro reaction, the experiment presented in Fig. 3 was repeated without adding radilabel to the reaction mixture. Reaction products were...
are pBR322 (the vector) or AAV DNA. As expected, there was a replication product in the reaction with the AAV/adenovirus coinfection extract, which hybridized to the AAV probe (Fig. 5). No \textit{Dpn} I-resistant species hybridized to the pBR322 probe. Thus, the \textit{in vitro} reaction appeared to mimic the \textit{in vivo} result by being highly specific for replication of the AAV sequences. This result was supported by also banding the products of a reaction in which BrdUTP was substituted for TTP in CsCl and blotting the individual fractions with either AAV or pBR322 probes after agarose gel electrophoresis; only AAV DNA hybridized to the hybrid density products (data not shown). In Fig. 5, the \textit{Dpn} I-resistant species again migrated near the top of the band representing the rescued species. Note that all the rescued species detected by the pBR322 probe appeared to migrate equivalently but that the AAV probe picked up a rescue species band with a slower component in lane 5 (Fig. 5B) and that the \textit{Dpn} I-resistant band in lane 6 corresponded to the slower component in lane 5.

\textbf{AAV Terminal Repeat Sequences Are Required.} To determine whether the AAV terminal repeat was necessary for replication, parallel replication reactions were performed by using plasmids with either intact terminal repeats or terminal repeats from which either 55 or 120 nucleotides had been deleted from each end (Fig. 2). Again, the products of each reaction were digested with \textit{Dpn} I. Deletion of 120 nucleotides from each end abolished replication (Fig. 3, lanes 9 and 10). Deletion of 55 nucleotides from each end prevented excision, but there were \textit{Dpn} I-resistant species migrating at the positions of forms II and III (lanes 11 and 12). Replication of the 55-base terminal deletion mutant was supported only by an extract from cells coinfected with both AAV and adenovirus (Fig. 6). Southern blots confirmed that both AAV and pBR322 sequences were present in the \textit{Dpn} I-resistant species (data not shown). We conclude that at least part of the terminal repeat is required to detect replication and that deletion of the terminal 55 bases abolishes excision and leads to replication of both AAV and pBR322.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Southern blot of DNA replication products. Products of four replication reactions with no radiolabel were fractionated by agarose gel electrophoresis and blotted onto nylon membranes. Blots were probed with either pBR322 (A) or AAV (B). Lanes: 1 and 2, products of a reaction mixture that contained an extract from uninfected HeLa cells; 3 and 4, extract from AAV-infected HeLa cells; 5 and 6, extract from AAV/adenovirus (Ad)-coinfected HeLa cells; 7 and 8, extract from adenovirus-infected HeLa cells. Products in lanes 2, 4, 6, and 8 were digested with \textit{Dpn} I. rp and \textit{ex} are as defined in Fig. 3.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Agarose gel electrophoresis of DNA replication reaction products. Lanes: 1 and 2, extract from infected HeLa cells; 3 and 4, extract from AAV-infected HeLa cells; 5 and 6, extract from AAV/adenovirus (Ad)-coinfected HeLa cells; 7 and 8, extract from adenovirus-infected cells. DNA substrate/template was pLB2904.}
\end{figure}
DISCUSSION

The in vitro assay described in this paper has characteristics comparable to those observed for AAV DNA replication in vivo. The ability to rescue the genome from an integrated state was consistent with the activation of the provirus observed in latent infection. Replication required an extract from cells coinfected by AAV and adenovirus and the presence of at least part of the palindromic region of the AAV inverted terminal repeat. Two observations were of particular interest. With an intact terminal repeat, Dpn I-resistant replication was only observed for the rescued AAV insert. Because it is likely that the rescued insert and the remaining vector sequence both had termini crosslinked by the AAV terminal hairpin (13), the selective replication of the AAV sequence is likely due to the presence of the sequence recognized by the nicking activity of the AAV rep 68/78 proteins (4). This sequence is not present in the rescued pBR322. The Dpn I-resistant AAV species appeared to migrate slightly more slowly on average than the pre-DpnI rescue product. This may indicate that replication also involved not only nicking at the hairpin but also extension to a normal double-helical terminus. A molecule extended in this fashion would migrate more slowly than one with shorter hairpin termini. The fact that excision occurred with all of the extracts regardless of detectable replication is consistent with earlier observations (13, 14). The second remarkable feature of the assay was the ability of a construct with 55 nucleotides deleted from both termini to replicate without excision. In this case, both AAV and pBR322 sequences were replicated. The accepted model of AAV DNA replication hypothesizes the terminal repeat in the hairpin configuration as the primer (Fig. 1). Yet it is difficult to picture this type of priming when the terminal 55 nucleotides are deleted and no rescue occurs. It seems more likely that another type of priming (e.g., internal origin) can be used. Interestingly, the putative internal origin requires the presence of at least part of the terminal palindrome.

An alternative model is that the priming for initiation of replication of the integrated DNA did not involve a DNA hairpin primer in either case. For a hairpin terminus to be used as a primer, the DNA has to be at least partially excised before onset of DNA replication. That this is possible is suggested by the data of both Gottlieb and Muzyczka (14) and Ward and Berns (13). However, in the latter study, addition of T antigen greatly enhanced the rescue process. The requirement of the intact terminal repeat palindrome for excision to occur suggests that conformation equivalent to a Holliday structure (25) may be required, and it seems likely that some active process along the DNA such as either the background type of repair synthesis seen in these experiments or actual DNA replication may have to occur first to induce a conformation (e.g., a cruciform or single-stranded DNA during lagging-strand replication) suitable for the resolution reaction responsible for rescue. Thus, it could be argued that the part of the terminal palindromic region after the 55-nucleotide terminal deletion contained the sequences sufficient for initiation of replication in either case. What was missing in the deletion mutant was a sufficient amount of palindrome to allow subsequent resolution. If resolution does occur, only AAV sequences are replicated; if it does not occur, replication continues on into the vector pBR322 sequences. The alternative model described here would suggest that AAV DNA replication in the integrated state initiates from an internal origin that requires some of the terminal repeat.

Only a limited amount of replication over an extended period of time (16 hr) was observed. We believe that the amount of replicated material seen after Dpn I digestion represented an underestimate and that the density-shift experiments probably most accurately reflected the true extent of replication (1% of total input). The results appear intermediate with respect to previously reported systems for in vitro replication in systems of animal virus DNAs. Adenovirus DNA replication is more extensive when infected cell extracts are used (24), whereas SV40 DNA replication in vitro was not achieved until sufficient active T antigen was purified (18). We suspect that use of sufficient AAV rep protein will enhance the in vitro AAV system in a comparable manner. Preliminary data support this notion (C. Leonard, G.H., P.W., and K.I.B., unpublished data). Development of the assay will permit detailed study of a mammalian replicon that is unique in that the template is initially in the integrated state and is subsequently excised. This assay is for a eukaryotic system likely to use two types of primers at the same origin of replication.

We wish to thank M. O'Donnell and J. Hurwitz for careful review of the manuscript and A. Beaton, C. Leonard, and M. Linden for helpful discussions. This work was supported by Grants AI22251 and AI26122 to K.I.B. from the U.S. Public Health Service.