JOURNAL OF VIROLOGY, July 1989, p. 3034–3039
0022-538X/89/073034-06$02.00/0

Vol. 63, No. 7

Identification of Nuclear Proteins That Specifically Interact with Adeno-Associated Virus Type 2 Inverted Terminal Repeat Hairpin DNA

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Received 24 January 1989/Accepted 21 March 1989

A palindromic hairpin duplex containing the inverted terminal repeat sequence of adeno-associated virus type 2 (AAV) DNA was used as a substrate in gel retardation assays to detect putative proteins that specifically interact with the AAV hairpin DNA structures. Nuclear proteins were detected in extracts prepared from human KB cells coinfected with AAV and adenovirus type 2 that interacted with the hairpin duplex but not in nuclear extracts prepared from uninfected, AAV-infected, or adenovirus type 2-infected KB cells. The binding was specific for the hairpin duplex, since no binding occurred with a double-stranded DNA duplex with the identical nucleotide sequence. Furthermore, in competition experiments, the binding could be reduced with increasing concentrations of the hairpin duplex but not with the double-stranded duplex DNA with the identical nucleotide sequence. S1 nuclease assays revealed that the binding was sensitive to digestion with the enzyme, whereas the protein-bound hairpin duplex was resistant to digestion with S1 nuclease. The nucleotide sequence involved in the protein binding was localized within the inverted terminal repeat of the AAV genome by methylation interference assays. These nuclear proteins may be likely candidates for the pivotal enzyme nickase required for replication or resolution (or both) of single-stranded palindromic hairpin termini of the AAV genome.

Adeno-associated virus type 2 (AAV), a dependovirus of human origin, requires coinfection with a helper virus (which can be adenovirus [1, 8, 16], herpesvirus [7], or vaccinia virus [32]) for its optimal growth, although helper-independent replication of AAV has recently been documented in certain human and hamster cell lines (41). It has been suggested, therefore, that the AAV-helper function may be provided by the host cell, induction of which may be augmented by the coinfecting helpervirus (2–4, 9), although the precise nature of the helper function still remains unknown.

The single-stranded DNA genome of AAV offers an excellent model system to investigate the mechanism of replication of linear DNA molecules (5, 28). The AAV genome contains inverted terminal repeats of 145 nucleotides which are palindromic and have been shown to serve as primers for AAV DNA replication (14, 23, 29, 31, 35, 37, 39). The replication of linear DNA molecules, however, presents an intriguing problem with regard to the resolution and repair of their 5′ ends. The model proposed by Cavalier-Smith (10) invokes the requirement of a hitherto unknown enzyme nickase in eucaryotic cells to overcome the problem of resolution and replication of palindromic termini. Interestingly, the precise requirement of certain structural and functional features of the model and the available experimental data on AAV DNA replication share a remarkable similarity (4, 23, 29, 31, 35). Although these experimental data strongly suggest the existence of a nickase, its detection and identification have thus far remained elusive.

In this pursuit, we utilized a palindromic hairpin duplex constructed in vitro from the AAV DNA inverted terminal repeats (35) as a substrate in gel retardation assays to examine whether AAV hairpin duplex DNA-binding proteins could be detected in infected cells. In this report, we present evidence for the existence of nuclear proteins in human KB cells coinfected with AAV and adenovirus type 2 (Ad2), that specifically interact with AAV inverted terminal repeat hairpin DNA. We also suggest that these proteins may be likely candidates for the putative nickase, the pivotal feature of the Cavalier-Smith model (10), as applicable to AAV DNA replication.

MATERIALS AND METHODS

Viruses and cells. Human AAV and Ad2 were kindly provided by K. I. Berns, Cornell University Medical College, New York, N.Y., and K. H. Fife, Indiana University School of Medicine, Indianapolis, Ind., respectively. Viruses were propagated in a human nasopharyngeal carcinoma established cell line KB monolayer cultures in Eagle minimal essential medium containing 10% fetal calf serum essentially as described previously (35–37).

Construction of AAV hairpin duplex DNA. A recombinant plasmid (p*sub*201) containing the entire AAV coding sequence flanked by the identical inverted terminal repeats (30) was a kind gift from R. J. Samulski, University of Pittsburgh, Pittsburgh, Pa. p*sub*201 DNA was digested to completion with restriction enzymes *Pvu*II and *Xba*I under the conditions specified by the supplier (Boehringer Mannheim Biochemicals, Indianapolis, Ind). A 191-base-pair *Pvu*II-*Xba*I DNA fragment containing the entire AAV inverted terminal repeat sequence was fractionated on and purified from a preparative 6% polyacrylamide gel essentially as described previously (37). The purified fragment was heat denatured (100°C, 6 min) and cooled rapidly to allow intrastrand hairpin formation, which was then used as a template in a reaction

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mixture containing 50 mM Tris hydrochloride (pH 7.8), 1 mM dithiothreitol, 0.01% bovine serum albumin, 10 mM $MgCl_2$, 100 mM KCl, all four deoxynucleoside triphosphates each at 200 μM, 100 μCi of [α-^{32}P]dCTP (specific activity, >3,000 Ci/mmol; Amersham Corporation, Arlington Heights, Ill.), and 5 U of the Klenow fragment of *Escherichia coli* DNA polymerase I (Pharmacia, Inc., Piscataway, N.J.) essentially as described previously (35). ^{32}P-labeled hairpin duplex DNA was separated from unincorporated [^{32}P]dCTP either by chromatography on Sephadex G-50 columns or by electrophoresis on 6% polyacrylamide gels followed by autoradiography. In some experiments, the hairpin duplex DNA was synthesized in the presence of unlabeled deoxynucleoside triphosphates and radiolabeled at 5′ ends by treatment first with calf intestinal phosphatase followed by T4 polynucleotide kinase in the presence of 200 μCi [γ-^{32}P]ATP essentially as described previously (24, 35).

Preparation of nuclear extracts. Human KB cells (~1×10^7 to 2×10^7) grown to confluence in 100-mm-diameter Petri dishes were mock infected, infected with AAV at a multiplicity of infection of 5, infected with Ad2 at a multiplicity of infection of 10, or coinfected with AAV and Ad2 as described elsewhere (37; P. Nahreini and A. Srivastava, Intervirology, in press). The cultures were incubated at 37°C in a CO_2 incubator (5% CO_2, 95% humidity) for 20 to 24 h. Nuclear extracts were prepared by two different methods exactly as described by Dignam et al. (12) and Muller (26). Total protein concentrations were determined by the method described by Lowry et al. (22), and portions were frozen in liquid nitrogen and stored at −80°C.

Gel retardation assays. DNA binding and gel electrophoresis were carried out essentially as described previously (33, 34). Briefly, binding assays were carried out in a total volume of a 20-μl solution containing 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1% Nonidet P-40, 1.6 μg of poly(dI-dC), 5 μg of nuclear extract, and 2 ng (~2×10^5 cpm) of the duplex DNA. The reaction mixtures were incubated at 20°C and loaded directly on nondenaturing 4% polyacrylamide slab gels (ratio of acrylamide-bisacrylamide, 30:1) containing 6.7 mM Tris base, 3.3 mM sodium acetate, and 1 mM EDTA. Gels were prerun for 2 to 4 h with constant buffer recirculation. Electrophoresis was carried out at 20 mA for 4 h. Gels were either fixed in 10% acetic acid—10% methanol (17) or exposed directly to X-ray film (XAR-5; Kodak Chemical Co., Rochester, N.Y.) and autoradiographed (38).

Methylation interference assays. Hairpin duplex DNA fragments labeled at their 5′ termini were partially methylated with dimethyl sulfate as described previously (24). After ethanol precipitation, the partially methylated hairpin duplex (~4×10^5 cpm) was incubated with nuclear extracts prepared from Ad2-AAV-coinfected KB cells in a standard DNA binding assay and electrophoresed on a 4% polyacrylamide gel as described above except that the gel was not fixed before autoradiography. The bound and free hairpin DNAs were eluted from the gel by the method described previously (24, 35). After β-elimination with 1.0 M piperidine and exhaustive lyophilizations (24), the labeled DNA samples were suspended in freshly deionized 90% formamide containing 0.1% bromophenol blue and 0.1% xylene cyanol, heated at 100°C for 3 min, and cooled rapidly. An equivalent number of counts was electrophoresed on a 12% polyacrylamide–8 M urea sequencing gel (24), and the gel was autoradiographed at −80°C.

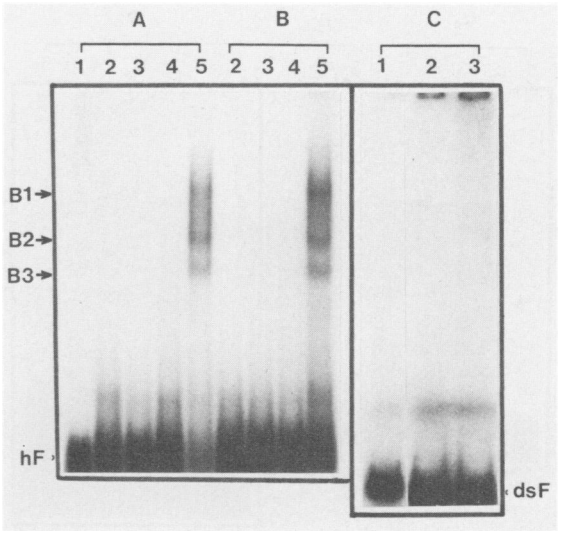

FIG. 1. Detection of AAV hairpin DNA-nuclear protein complex formation. ^{32}P-labeled AAV hairpin DNA constructed in vitro and end-labeled double-stranded duplex DNA were incubated with nuclear extracts prepared from uninfected and infected human KB cells and fractionated on low-ionic-strength polyacrylamide slab gels as described in Materials and Methods. (A) Unbound hairpin DNA (denoted hF; lane 1) was incubated with nuclear extracts from uninfected (lane 2), AAV-infected (lane 3), Ad2-infected (lane 4), and Ad2-AAV-coinfected (lane 5) KB cells at 20°C for 15 min. (B) Same as in panel A except that the reactions were carried out at 20°C for 30 min. B1, B2, and B3 represent the DNA-protein complexes formed with nuclear proteins prepared from Ad2-AAV-coinfected KB cells. (C) Unbound double-stranded duplex DNA (denoted dsF; lane 1) showed no complex formation with nuclear proteins prepared from either Ad2-infected (lane 2) or Ad2-AAV-coinfected (lane 3) KB cells.

RESULTS

Formation of AAV hairpin DNA-protein complexes in vitro with nuclear extracts prepared from Ad2-AAV-coinfected KB cells. Gel retardation assays have been successfully used in a variety of biological systems to identify proteins that specifically interact with a given DNA sequence (13, 17, 19, 33, 34). We used this assay to investigate whether eucaryotic cells contain nuclear proteins that specifically interact with the AAV hairpin DNA structure. ^{32}P-labeled hairpin duplexes containing the entire inverted terminal repeat sequence of AAV were incubated with uninfected and infected KB cell nuclear extracts in vitro prepared by the method of Muller (26) and analyzed on low-ionic-strength gels as described in Materials and Methods. The results of such an experiment are shown in Fig. 1.

Two sets of assays were carried out. In the first set, nuclear extracts were incubated with a nonspecific competitor DNA [poly(dI-dC)] at 0°C for 15 min followed by the addition of ^{32}P-labeled hairpin DNA and subsequent incubation for 15 min at 20°C (Fig. 1A). In the second set, nuclear extracts were preincubated with poly(dI-dC) for 30 min at 20°C followed by the addition of ^{32}P-labeled hairpin DNA and incubation for an additional 30 min at 20°C (Fig. 1B). It is interesting to note that the unbound hairpin DNA (denoted hF; Fig. 1A, lane 1) showed no binding with nuclear proteins in uninfected cells, AAV-infected cells, or Ad2-infected cells (Fig. 1A and B, lanes 2 to 4, respectively). Three distinct complexes (B1, B2, and B3) were clearly visible with nuclear proteins prepared from Ad2-AAV-coinfected KB cells (Fig.

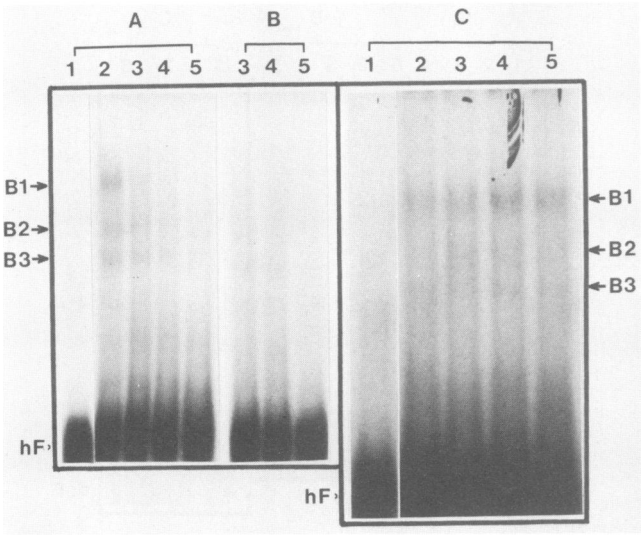

FIG. 2. Competition binding analysis for the specificity of the hairpin DNA-protein complex formation. Standard binding assays were carried out as described in Materials and Methods except that the ratios of either the unlabeled AAV hairpin or double-stranded duplex DNA and the ^{32}P-labeled AAV hairpin DNA were progressively increased. (A) Unbound hairpin DNA is indicated in lane 1. The hairpin DNA-protein complex formation (lane 2) was gradually reduced with the inclusion of nonradioactive, complete hairpin DNA at a ratio of 1:1 (lane 3), 1:3 (lane 4), and 1:5 (lane 5). (B) Same as in panel A except that the incomplete hairpin DNA was used as a competitor at the ratios listed above. (C) Unbound hairpin DNA is indicated in lane 1. The hairpin DNA-protein complex formation (lane 2) was not affected by the double-stranded duplex DNA with identical nucleotide sequence at ratios of 1:1 (lane 3), 1:3 (lane 4), and 1:5 (lane 5).

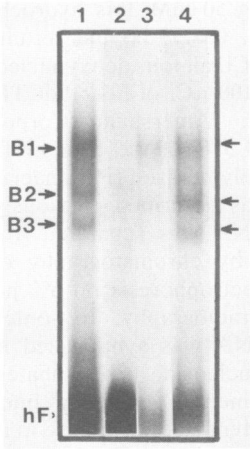

FIG. 3. S1 nuclease sensitivity and protection analysis of the hairpin DNA-protein complex formation. The assays were carried out as described in the text. Lanes: 1, hairpin DNA-protein complex formation under standard assay conditions; 2, unbound hairpin DNA treated with S1 nuclease; 3, pretreatment of the hairpin DNA with S1 nuclease followed by incubation with the nuclear extract; 4, hairpin DNA-protein complex formation followed by digestion with S1 nuclease.

1A and B, lanes 5) that interacted with the AAV hairpin DNA. The binding was specific for nuclear proteins prepared from Ad2-AAV-coinfected KB cells and survived numerous freeze-thaw cycles of the extract over a period of more than 1 year. Identical results were obtained (data not shown) with nuclear extracts prepared from Ad2-AAV-coinfected KB cells by the method described by Dignam et al. (12). These results strongly suggest the existence of nuclear proteins that are host cell proteins induced by Ad2-AAV-coinfection, AAV proteins induced by Ad2 infection, or a combination thereof. It is also of interest to note (Fig. 1C) that a double-stranded DNA duplex (denoted dsF; Fig. 1C, lane 1) with a nucleotide sequence identical to that of the hairpin duplex showed no such complex formation either with nuclear extracts prepared from Ad2-infected cells (Fig. 1C, lane 2) or Ad2-AAV-coinfected KB cells (Fig. 1C, lane 3). These data thus suggest the specificity of binding of nuclear proteins present in Ad2-AAV-coinfected KB cells only to the AAV inverted terminal repeat sequence in the hairpin configuration. The rest of the experiments were carried out with nuclear extracts from Ad2-AAV-coinfected KB cells prepared by the method of Muller (26).

Specificity of binding by competition analysis. Although binding specificity to the AAV hairpin duplex was indicated, we sought to extend these studies in which binding experiments were carried out in the presence of increasing concentrations of either complete hairpin duplexes, incomplete hairpins (heat-denatured fragments in the absence of repair by DNA polymerase), or double-stranded duplexes. The results of such a competition assay are shown in Fig. 2.

It is evident that the hairpin duplex (Fig. 2, lanes 1) once again showed three distinct complexes with nuclear proteins from Ad2-AAV-coinfected KB cells (Fig. 2, lanes 2), but the extent of complex formation was gradually decreased with increasing concentrations of unlabeled complete hairpin duplexes (Fig. 2A, lanes 3 to 5), as well as with incomplete hairpin duplexes (Fig. 2B, lanes 3 to 5) and was virtually eliminated at a labeled-to-unlabeled hairpin ratio of 1:5. Under identical conditions, however, the complex formation between the hairpin duplex and nuclear proteins (Fig. 2C, lane 2) was not affected by increasing concentrations of unlabeled double-stranded duplex DNA (Fig. 2C, lanes 3 to 5). These results further establish that the observed complex formation is specific for the AAV inverted terminal repeat sequence only when it is present in a hairpin configuration. The rest of the studies were carried out with the complete hairpin duplex DNA.

S1 nuclease sensitivity and protection analysis. Previous studies have documented that 125 of 145 nucleotides in the AAV inverted terminal repeats are involved in maximum base pairing to generate a T-shaped hairpin structure (23), in which there are three regions that contain a total of seven unpaired nucleotides. It was, therefore, of significant interest to examine whether removal of these single-stranded regions would affect the complex formation. The hairpin duplex was first treated with S1 nuclease under conditions in which double-stranded DNA is relatively resistant to digestion by this enzyme (11) followed by complex formation in standard assays with nuclear extracts from Ad2-AAV-coinfected KB cells. In addition, the DNA-protein complex was subsequently treated with S1 nuclease to examine the extent of the susceptibility of the hairpin DNA to digestion with this enzyme. These results are depicted in Fig. 3.

As can be seen, the complex formation between the AAV hairpin duplex and the nuclear proteins was observed under standard assay conditions (Fig. 3, lane 1), but the prior digestion of the hairpin DNA (Fig. 3, lane 2) with S1 nuclease significantly reduced the extent of complex forma-

tion with nuclear proteins from Ad2-AAV-coinfected KB cells (Fig. 3, lane 3). In contrast, the protein-bound hairpin DNA complexes were resistant to digestion with S1 nuclease (Fig. 3, lane 4). These data suggest that the T-shaped structure without the hairpin bends is not recognized by the nuclear proteins, which in turn protect these single-stranded regions from digestion with S1 nuclease, underscoring the specificity of binding to the AAV hairpin DNA.

Methylation interference assays for binding site analysis. It was of significant interest to next localize the nucleotide sequence in the AAV hairpin DNA structure involved in the complex formation with the nuclear proteins. This was carried out by a chemical footprinting technique (24) to identify the G residues involved in the binding by subjecting them to methylation prior to binding. Since methylation of G residues corresponds directly with subsequent cleavage, a quantitative measure of cleavage at G residues in both free and bound hairpin DNAs would be expected to indicate the G residues involved in the complex formation. For example, since prior methylation of G residues would preclude their involvement in protein binding, DNA molecules containing these G residues would be eliminated from the complex. Correspondingly, hairpin molecules involved in protein binding would exhibit diminished cleavage intensity at these G residues. 5′ end labeled, partially methylated hairpin DNA was used as a substrate in complex formation with nuclear proteins from Ad2-AAV-coinfected KB cells as described in Materials and Methods. Both bound and free DNA fragments were recovered from a preparative gel, cleaved at G residues, and analyzed on a sequencing gel to obtain a footprint. Such a footprint for one of the lower-molecular-weight complexes is shown in Fig. 4.

Figure 4A depicts the relative efficiencies of cleavage at G residues in the unbound hairpin DNA (lane 1) and that involved in the complex formation (lane 2). It is clear that the cleavage efficiency in the bound hairpin DNA was significantly reduced along almost the entire sequence, suggesting that most of the G residues in the AAV inverted terminal repeat sequence were involved in the complex formation. This is further illustrated in the schematic representation of the AAV inverted terminal repeat sequence (Fig. 4B). The palindromic terminus forms a T-shaped hairpin structure in which a large palindromic stretch (AA′) forms the stem and two smaller internal palindromes (BB′ and CC′) form the crossarms of the T (23). The remainder of the unpaired 20 nucleotides is designated as sequence D. Evaluation of these data revealed that except for G residues in sequence D and in the smaller palindrome BB′ (highlighted by arrowheads), the entire AAV hairpin DNA sequence was involved in the complex formation. Similar results have been obtained (N. Muzyczka, personal communication) in studies utilizing DNase I footprinting assays.

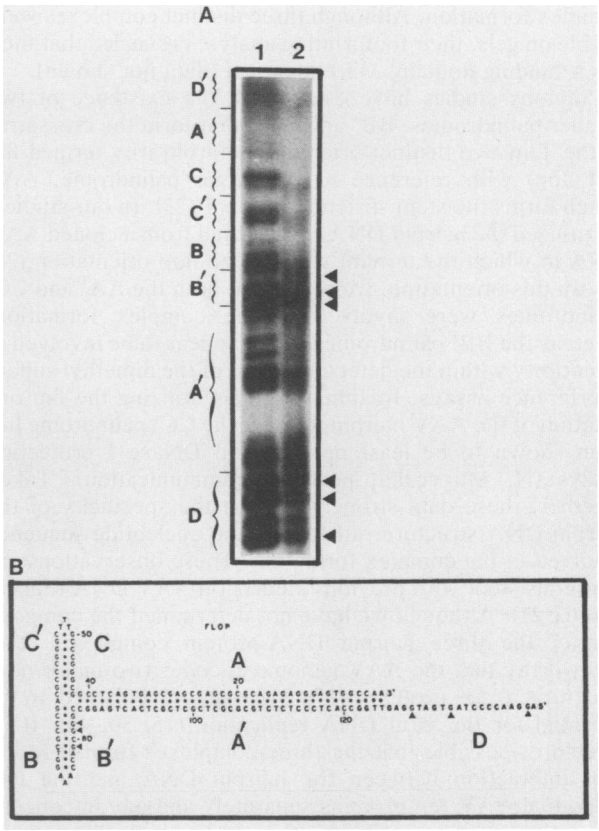

FIG. 4. Footprinting analysis with dimethyl sulfate interference assays. (A) ^{32}P-end-labeled AAV hairpin DNA was partially methylated, incubated with nuclear extracts, and fractionated on a polyacrylamide gel, and both free and bound DNA fragments were eluted and purified as described in Materials and Methods. After cleavage with piperidine, equivalent amounts of radioactivity of free (lane 1) and bound (lane 2) hairpin DNA samples were fractionated on a 12% polyacrylamide–8 M urea sequencing gel. (B) Nucleotide sequence and organization of the AAV inverted terminal repeat in the flop orientation (23). The G residues not involved in the complex formation are identified by arrowheads (◀).

DISCUSSION

The apparently defective nature of AAV has been an intriguing problem (for reviews, see references 3, 4, and 9). Two sets of requirements have been enumerated for the productive growth and replication of AAV, coinfection with a helper virus (3) and S phase of the cell cycle (41), and several adenovirus-encoded gene products involved in AAV helper function have been identified (9, 18). However, their underlying molecular mechanisms still remain elusive. Moreover, the precise nature of the host cell component required for AAV replication and presumably expressed during the S phase of the cell cycle remains virtually unknown.

Although AAV DNA replication proceeds through a double-stranded DNA intermediate (14, 39), the possibility that the single-stranded nature of the AAV genome may contribute to the apparent problem in a milieu in which the genomic DNAs of both the helper virus and the host cell are double stranded prompted us to investigate whether putative proteins exist in the host cell that specifically interact with the single-stranded AAV hairpin termini. The hairpin termini which play a critical role as primers for AAV DNA replication (2–4, 35) also pose a problem with regard to their resolution and repair (10). These studies could thus be expected to document the existence of a putative enzyme, nickase, which is capable of circumventing this problem based on the Cavalier-Smith model for the replication of linear, single-stranded DNA molecules (10).

Indeed, specific proteins were detected in nuclear extracts prepared from Ad2-AAV-coinfected human KB cells that interacted with the AAV hairpin DNA but not with the double-stranded duplex DNA containing the same inverted terminal repeat sequence, suggesting the possibility that the binding was structure rather than sequence specific. The S1 nuclease analysis further indicated that the single-stranded hairpin bends in the T-shaped structure were critical in the

complex formation. Although three distinct complexes were visible on gels, their footprinting analyses revealed that their DNA binding domains were identical (data not shown).

Previous studies have established the existence of two smaller palindromes, BB′ and CC′, that form the crossarms of the T in two distinct orientations (arbitrarily termed flip and flop) with reference to the larger palindrome, AA′, which forms the stem structure of the T (23). In our studies, we utilized the hairpin DNA constructed from a cloned AAV DNA in which the termini were in the flop orientation (29, 30). In this orientation, we noted that both the AA′ and CC′ palindromes were involved in the complex formation, whereas the BB′ palindrome did not appear to be involved in its entirety within the detection limits of the dimethyl sulfate interference assays. In similar studies utilizing the flip orientation of the AAV hairpin termini, the CC′ palindrome has been shown to be least protected in DNase I protection assays (N. Muzyczka, personal communication). Taken together, these data strongly suggest the specificity of the hairpin DNA structure rather than the nucleotide sequence involved in the complex formation. These observations are thus consistent with previous studies on AAV DNA replication (6, 21). Although we have not determined the composition of the three hairpin DNA-protein complexes, it is noteworthy that the AAV genome encodes two major nonstructural (*rep*) proteins (25, 37) that are believed to be essential for the viral DNA replication (15, 30, 40). It is, therefore, possible that the three complexes formed represent interaction between the hairpin DNA and the two individual AAV *rep* proteins separately and one in concert, respectively. Alternatively, the higher-molecular-weight complexes may also include cooperative binding of various other viral and host cell factors that are required for AAV DNA replication. The fact that these complexes are not observed in nuclear extracts prepared from uninfected, AAV-infected, or Ad2-infected KB cells lends further support to this hypothesis. Indeed, the hairpin-interacting proteins have recently been shown to be the AAV *rep* proteins (N. Muzyczka, personal communication). Interestingly, previous studies have identified several proteins that either are linked to the 5′ ends of the replicative forms of an autonomously replicating parvovirus DNA (27) or interact specifically with the single-stranded regions of the replicating adenovirus DNA (20). Their close association with these structures, therefore, implies their role in the process of initiation of DNA replication with, or resolution of, hairpin DNA structures, or both. It is intriguing, however, that despite the apparent noninvolvement of the double-stranded duplex DNA in the complex formation with these nuclear proteins, the replication of reannealed AAV DNA either from mature virions or from recombinant plasmids upon transfection and subsequent rescue is quite efficient (6, 15, 21, 29–31, 40). Whether one or more of these nuclear proteins also catalyze the conversion of duplex termini of the AAV DNA into a hairpin configuration in vivo remains an open question. At the very least, the gel retardation assays described here should permit isolation, purification, and characterization of individual viral and host cell proteins or factors involved in AAV DNA replication.

Finally, the identification of nuclear proteins that specifically interact with hairpin DNA molecules may prove useful in gaining an insight into the molecular mechanisms underlying eucaryotic chromosomal telomere DNA replication. It is now of significant interest to examine the associated enzymatic and catalytic activities of the DNA-protein complexes formed between the AAV hairpin termini and the nuclear proteins in Ad2-AAV-coinfected human KB cells.

ACKNOWLEDGMENTS

We thank R. J. Samulski for the kind gift of plasmid p*sub*201 and for helpful suggestions. We are grateful to K. I. Berns and K. H. Fife for providing the AAV2 and Ad2 stocks, respectively. We also thank N. Muzyczka for communicating to us his unpublished results, and A. Roman and R. Bockrath for a critical review of the manuscript. The expert secretarial assistance of Stephanie Moore during the preparation of the manuscript is gratefully acknowledged.

This research was supported in part by Public Health Service grant AI-26323 from the National Institutes of Health, a grant-in-aid from the American Heart Association-Indiana Affiliate, and a grant from the Phi Beta Psi Sorority to A.S.

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