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Evidence for Covalent Attachment of the Adeno-Associated Virus (AAV) Rep Protein to the Ends of the AAV Genome

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We have demonstrated that when the covalently joined ends of linear adeno-associated virus (AAV) DNA are resolved in vitro, the virus-encoded Rep protein becomes covalently attached to the 5′ ends of the DNA. The covalent bond is between a tyrosine residue of the AAV Rep protein and a 5′ phosphate of a thymidine residue in the AAV genome. Only the Rep protein encoded by the AAV p5 promoter, Rep68, was capable of becoming covalently attached to the ends of the AAV genome; the Rep proteins encoded by the p19 promoter were not. We also investigated some of the requirements for the complete in vitro resolution reaction. Inhibitor studies suggested that terminal resolution required DNA polymerase δ, ATP, and the deoxyribonucleoside triphosphates but did not require the remaining ribonucleoside triphosphates, DNA polymerase α, RNA polymerase II, or topoisomerases I and II. Finally, purified AAV Rep68, when added to the crude cytosol from uninfected HeLa cells, was sufficient for resolution. This suggested that terminal resolution relies on host enzymes and the virus-encoded p5 Rep proteins.

Adeno-associated virus (AAV) contains a single-stranded DNA genome of 4,680 bases (5, 68). Efficient replication of the AAV chromosome requires the presence of a helper virus (5). The most efficient helper viruses are herpesviruses or adenoviruses, but their proteins do not appear to be directly involved in the replication of AAV DNA (5, 11, 47, 64, 78). Two AAV genes are required for AAV DNA replication. The first is the terminal repeat which is required for, in addition to DNA replication (62, 65), integration, rescue, and packaging of AAV genomes (46, 61). The second is the *rep* gene (28, 73) which codes for a family of four nonstructural proteins: Rep78, Rep68, Rep52, and Rep40 (48, 68). The two larger Rep proteins (Rep78 and Rep68) are synthesized from the mRNAs initiated at the AAV p5 promoter, while the transcripts for the smaller Rep proteins are initiated at the p19 promoter (25, 26, 36, 44, 48, 68). Mutations which affect only the p5 Rep proteins are defective for viral DNA replication and the transactivation of viral gene expression (28, 33, 74). The smaller p19 Rep proteins do not appear to have a role in terminal resolution (14).

The model for AAV DNA replication (6, 13, 42, 43, 70) predicts that the terminal hairpins prime DNA synthesis to produce an intermediate which is covalently joined at one end (Fig. 1, step a). Following this, a site-specific and strand-specific nick is made at the terminal resolution site (*trs*) (6, 32, 42, 43, 59, 66). This produces a new 3′ OH primer that allows DNA synthesis to repair the covalent end (Fig. 1, steps b to d) (6, 66). The end of the resolved open duplex intermediate then undergoes denaturation to reform the terminal hairpins and to prime strand displacement synthesis (Fig. 1, steps e and f).

Recently, we developed an in vitro assay in which AAV molecules which have covalently joined ends can be resolved to an open duplex form (66) (Fig. 1, steps b to d). The resolution reaction required a crude extract prepared from cells infected with adenovirus and AAV. During the course of this in vitro reaction, it appeared that a protein became covalently attached to the 5′ ends of the product DNA (66). Subsequently, we developed another in vitro assay which was designed to detect the site-specific and strand-specific nicking at the *trs* site (32) (Fig. 1, step b). The *trs* endonuclease reaction required purified preparations of the AAV-coded nonstructural protein Rep68, and the DNA product of this reaction also appeared to be covalently attached to a protein (32).

In this report, we investigate the identity of the protein covalently joined to the 5′ ends of in vitro-resolved or -nicked AAV DNA. We present direct evidence that the p5 Rep protein, Rep68, is covalently attached to the 5′ ends of the AAV genome in both types of in vitro reactions and that this attachment occurs between a tyrosine in the protein and a 5′ phosphate of a thymidine residue in AAV DNA. We also investigate some of the other requirements for the complete in vitro resolution reaction.

MATERIALS AND METHODS

Plasmid and viral DNA. Plasmid p*sub*201(+) has been described previously (60); the particular isolate used was SSV16. pSM620 has also been described previously (59). Preparation of plasmid DNA was carried out by standard procedures (45). NE substrate DNA was made as described elsewhere (66).

Radiolabeled NE substrate. p*sub*201(+) plasmid DNA (10 μg) was digested with 20 U of *Pvu*II at 37°C for 6 h. Following phenol extraction and ethanol precipitation, the digested products were dissolved in water. The no-end (NE) substrate was synthesized in a reaction mixture that contained (in 30 μl) 10 μg of *Pvu*II-digested p*sub*201(+) DNA, 66 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA (pH 7.5), and 60 U of *Escherichia coli* exonuclease III (ExoIII). The reaction mixture was incubated at 37°C for 7 min, and the ExoIII was inactivated by heating at 75°C for 15 min. The reaction mixture was adjusted to 60 μl containing 66 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA (pH 7.5), the four deoxynucleoside triphosphates (0.3 mM each), [α-^{32}P]TTP (3,000 Ci/mmol), 1 mM ATP, 10 mM dithiothreitol, 2 U of T4 DNA polymerase, and 400 U of T4 DNA

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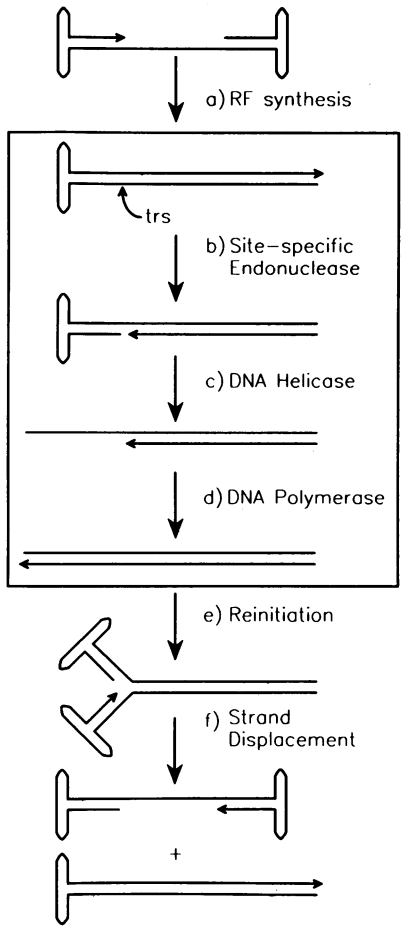

FIG. 1. Model for AAV DNA replication. Individual steps are described in the text. RF, Replicative form.

ligase. The mixture was incubated at 37°C for 1.5 h, and enzymatic activity was again terminated by heating the mixture at 75°C for 15 min. Following this, the products of the repair reaction were digested to completion with ExoIII (400 U) at 37°C for 2 h. NE DNA was separated from unincorporated radioactivity by gel filtration. Enzymes were purchased from New England BioLabs, and ^{32}P-labeled nucleotides were purchased from ICN.

Cell extracts. Crude cytoplasmic extracts were prepared as described previously (66) from HeLa cells grown in suspension culture (5×10^5 cells per ml) in Dulbecco modified Eagle medium with 10% calf serum. The cells were mock infected or infected with either adenovirus type 2 alone (multiplicity of infection = 5) or adenovirus type 2 and AAV type 2 (AAV2) (multiplicity of infection = 20). The cells were harvested at 35 h postinfection. The extracts had a protein concentration of 10 mg/ml as assayed by the Bradford assay (7).

Terminal resolution assay. The standard reaction mixture (without presynthesis) contained (in 50 μl) 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; pH 7.5); 7 mM $MgCl_2$; 0.5 mM dithiothreitol; 100 μM (each) dCTP, dGTP, dATP, and dTTP; 4 mM ATP; 200 μM (each) CTP, GTP, and UTP; 40 mM creatine phosphate; 1 μg of creatine phosphokinase; 0.5 μg of radiolabeled NE substrate; and 16 μl of cytoplasmic extract. If unlabeled NE substrate was used, [α-^{32}P]dCTP (3,000 Ci/mmol) was added to the reaction mixture. A time course of the in vitro resolution reaction was performed to determine the optimal time which would yield the most resolved product. Resolution products were detected at the earliest time point (30 min) and increased linearly for 2.5 h. After 2.5 h, there was little change in the yield of product. The standard reactions were incubated for 3 h at 37°C. To analyze the DNA products, the reaction mixture was diluted twofold with water and the reaction was terminated by the addition of EDTA and sodium dodecyl sulfate (SDS) to final concentrations of 10 mM and 0.2%, respectively. The reaction products were then treated with proteinase K (20 μg) at 37°C for 1 h, extracted with phenol and chloroform (1:1), precipitated with ethanol, and dissolved in water. Polyacrylamide gel electrophoresis was performed in TBE buffer (Tris-borate-EDTA); agarose gel electrophoresis was performed in TAE buffer (Tris-acetate-EDTA). Autoradiography was carried out at room temperature (without an intensifying screen) or at −70°C (with an intensifying screen).

Terminal resolution reaction requirements. Various enzyme inhibitors were added to the in vitro resolution reaction to determine which cellular enzymes were required. Aphidicolin (Sigma) dissolved in dimethyl sulfoxide was added to a final concentration of 10 μg/ml to determine the DNA polymerase requirements. α-Amanitin (Calbiochem) dissolved in water was added to a final concentration of 10 μg/ml to inhibit RNA polymerase II. Camptothecin and VM26 (gifts of P. Tegtmeyer), dissolved in dimethyl sulfoxide, were added to a final concentration of 200 μM to inhibit topoisomerase I or II, respectively (79).

Isolation of Rep68 (I) fraction. The purified Rep fraction used in these studies was Rep68 (I). It is identical to the fraction described previously (32). Rep68 (I) was purified from a 0.2 M NaCl extract of HeLa cell nuclei infected with adenovirus and AAV by chromatography on phenyl Sepharose, DEAE cellulose, and single-stranded DNA agarose.

***trs* endonuclease assay.** The standard *trs* endonuclease reaction mixture contained (in a volume of 20 μl) 25 mM HEPES-potassium hydroxide (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM ATP, 0.2 μg of BSA, radiolabeled NE substrate, and 5 U of Rep68 (I). The reaction began with the addition of $MgCl_2$ and was incubated for 60 min at 37°C as described previously (32).

Analysis of protein attached to labeled NE DNA. The in vitro resolution reaction products and *trs* endonuclease reaction products were treated with 200 U of micrococcal nuclease (Boehringer Mannheim) and 2 μg of DNase I (Sigma) in the presence of 2.5 mM $CaCl_2$ for 2 h at 37°C. An equal volume of 2× protein sample buffer (containing SDS and 2-mercaptoethanol) was added, and the samples were boiled, centrifuged, and loaded onto a 10% SDS-polyacrylamide gel (34). After electrophoresis, the gels were dried and autoradiographed. Immunoblotting was performed as described elsewhere (31). Protein molecular weight standards were purchased from Sigma and Amersham.

Paper electrophoresis. The in vitro resolution reaction products were treated with 200 U of micrococcal nuclease and 2 μg of DNase I in the presence of 2.5 mM $CaCl_2$ for 2 h at 37°C. The samples were then digested with 80 μg of pronase E (Sigma) in the presence of 10 mM EDTA and 0.2% SDS for 2 h at 37°C. After lyophilization, 150 μl of constant-boiling 6 N HCl (Pierce) was added to each sample and the samples were incubated for 1.25 h at 110°C. The hydrolyzed products were lyophilized and dissolved in 10 mM Tris hydrochloride (pH 8) containing 1-mg/ml *O*-phosphotyrosine, *O*-phosphoserine, and *O*-phosph threonine markers

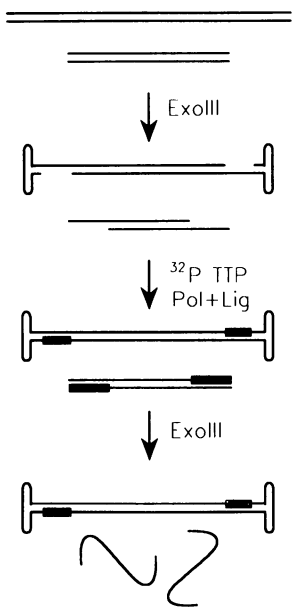

FIG. 2. Synthesis scheme of radiolabeled NE substrate. The starting material is a *Pvu*II digest of p*sub*201(+) plasmid DNA. *Pvu*II digestion exactly separates vector DNA (shorter lines) from AAV sequences (longer lines). The boxes indicate regions of ^{32}P-TMP incorporated during gap repair. Pol, T4 DNA polymerase; Lig, T4 DNA ligase; ^{32}P TTP, [α-^{32}P]TTP.

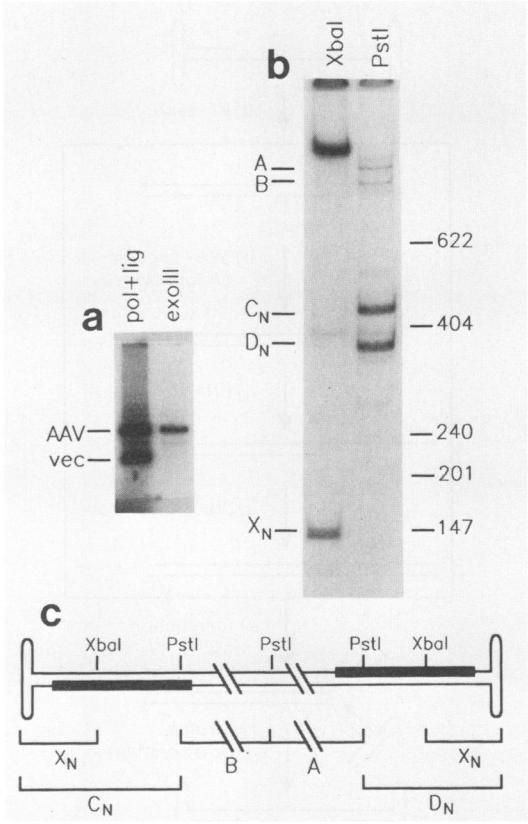

FIG. 3. Analysis of radiolabeled NE substrate. (a) Products during the synthesis of radiolabeled NE DNA. Portions of the products from the gap repair reaction (pol+lig) and from the final ExoIII reaction were separated on a 1% agarose gel. AAV and vec indicate the fragments containing AAV and vector sequences, respectively. (b) Location of the label in radiolabeled NE substrate. Radiolabeled NE substrate was digested with *Xba*I or *Pst*I, and the products were separated on a 4% nondenaturing polyacrylamide gel. DNA size markers (in base pairs) are indicated on the right. The terminal *Pst*I and *Xba*I fragments (for *Pst*I, C_N and D_N; for *Xba*I, X_N) are indicated on the left, and their locations in the AAV genome are indicated in panel c. Panel c also indicates the approximate locations of the incorporated radioactivity (boxes) in the NE substrate.

(Sigma). The samples were then spotted onto Whatman 3MM paper and electrophoresed in one dimension at pH 3.5 in pyridinium acetate (29). Visualization of the phosphoamino acid markers was carried out by spraying them with 0.5% ninhydrin dissolved in ethanol.

DNA hybridization. The in vitro resolution reactions were carried out with unlabeled NE substrate and unlabeled nucleotides. The products were digested with proteinase K, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol. The products were then digested with *Pst*I and electrophoresed on a 4% acrylamide gel. Electrophoretic transfer of the DNA to a Zeta-Probe (Bio-Rad) membrane was carried out in TAE buffer. The membrane was blocked and hybridized according to manufacturer's instructions. Nick-translated pSM620, a plasmid harboring a wild-type AAV genome (59), was used as the probe.

RESULTS

Synthesis of radiolabeled no-end substrate. As mentioned earlier, during both the terminal resolution reaction (with crude extracts) and the *trs* endonuclease assay (with purified Rep68), a protein was apparently attached to the 5′ ends of the product DNA at the terminal resolution site. If this protein was Rep68, it should be possible to show the transfer of a radiolabeled nucleotide from the DNA substrate to Rep68 in both reactions. To demonstrate this, it was necessary to synthesize radiolabeled NE substrate.

NE substrate which was radiolabeled at the *trs* site was synthesized as described in the legend to Fig. 2. During the synthesis of NE substrate (66), ExoIII was used to digest each strand of a duplex AAV DNA molecule at both 3′ ends. This allowed the terminal hairpins to form and left a gap at each end of the molecule which extended from the 5′ end of the molecule to a variable 3′ end left by ExoIII (Fig. 2). The terminal resolution sites (where the site-specific cuts occurred) were located within these gaps. Therefore, to synthesize radiolabeled NE substrate, the gaps were repaired by T4 DNA polymerase and T4 DNA ligase in the presence of [α-^{32}P]TTP. This procedure ensured that the thymidine residues at the *trs* sites (5′-GGAGT^{125} ↓ ^{124}TGGCC-3′), which are the primary targets for site-specific cleavage, would be labeled in the substrate (23, 32, 66). Following treatment with T4 DNA polymerase and T4 DNA ligase, the products were treated with an excess of ExoIII to remove DNA that had not been converted to NE substrate (Fig. 2 and 3a). To confirm that the NE DNA had been correctly labeled, the NE substrate was digested with *Xba*I or *Pst*I and analyzed on a 4% acrylamide gel to determine the location of the incorporated radioactivity. As expected (Fig. 3b and c), most of the radioactivity was incorporated into the terminal *Xba*I fragments (X_N) and into the proximal *Pst*I fragments from the left and right sides of the genome (C_N and D_N, respectively). Relatively little of the radioactivity was incorporated into the internal *Pst*I A and B fragments.

Covalent attachment of the p5 Rep68. To demonstrate

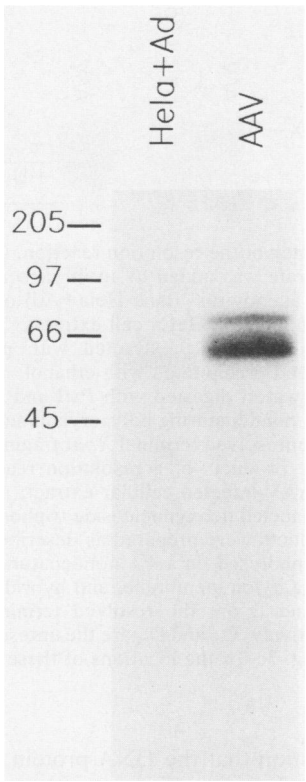

FIG. 4. AAV-specific ^{32}P-labeled protein. Radiolabeled NE substrate was added to resolution reactions by using extracts prepared from adenovirus- (lane Hela+Ad) or adenovirus-plus-AAV- (lane AAV) infected HeLa cells. The products of these reactions were digested with micrococcal nuclease and DNase I and electrophoresed on a 10% SDS-polyacrylamide gel. Protein molecular mass standards (in kilodaltons) are indicated on the left.

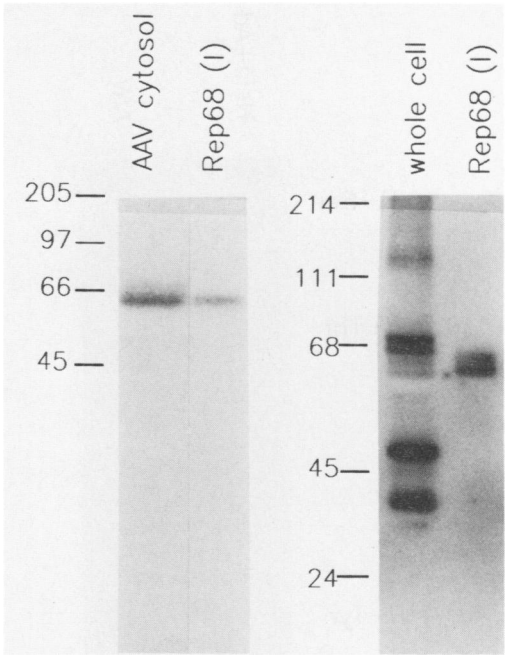

FIG. 5. Comparison of the products from the terminal resolution and *trs* endonuclease reactions. (Left panel) Radiolabeled NE DNA was used as the substrate in the terminal resolution reaction with crude cytosol (AAV cytosol) or the *trs* endonuclease reaction with purified Rep68 [Rep68 (I)]. The reaction products were digested with DNase I and micrococcal nuclease and electrophoresed on a 10% SDS-polyacrylamide gel. Protein molecular mass standards (in kilodaltons) are indicated on the left. (Right panel) Immunoblot of an adenovirus-plus-AAV-infected whole-cell extract and the purified AAV Rep68 fraction [Rep68 (I)] with polyclonal anti-Rep antibody. The proteins were separated on an 8% SDS-polyacrylamide gel; protein molecular mass standards (in kilodaltons) are indicated on the left. The Rep proteins in the Rep68 (I) fraction migrated slightly slower than the Rep proteins in the whole-cell extract because glycerol was present in the Rep68 (I) fraction.

clearly that a protein is covalently bound to the ends of AAV DNA after terminal resolution and to determine the size of the protein, an experiment was done in which radioactivity was transferred from NE DNA to the protein. Radiolabeled NE substrate was incubated with adenovirus-infected or adenovirus- and AAV-infected cytoplasmic HeLa cell crude extracts in a standard in vitro resolution reaction. The products of the reaction were then digested with DNase I and micrococcal nuclease and separated on a 10% SDS-polyacrylamide gel (Fig. 4). Micrococcal nuclease hydrolyzes DNA to leave products with 3′ phosphate residues so that if the digestion were complete, only one phosphate group would remain attached to the protein following digestion (40, 58).

In the case of the adenovirus-plus-AAV-infected crude extract (Fig. 4), most of the radioactivity was associated with a protein that had an apparent molecular weight identical to that of Rep68. The theoretical molecular sizes of the Rep proteins are 71, 61, 45, and 35 kDa (68), but they are commonly known by their apparent molecular sizes on high-percentage protein gels, namely, Rep78, Rep68, Rep52, and Rep40 (48). The adenovirus-plus-AAV-infected cytoplasmic extract used for the in vitro resolution reactions contained Rep68, Rep52, and Rep40, as determined by immunoblot analysis, but little, if any, Rep78 (data not shown). Rep78 has been shown to reside primarily in the nucleus (48) and was found to be underrepresented in the cytoplasmic extract used here (D.-S. Im and N. Muzyczka, unpublished data). The ratio of the Rep68, Rep52, and Rep40 proteins in the extract was 1:10:10 (data not shown). In contrast to Rep68, which was readily labeled during the in vitro resolution reaction, the Rep proteins synthesized by the p19 promoter (Rep52 and Rep40) were not labeled. Apparently, Rep52 and Rep40 were unable to attach covalently to the 5′ ends of the AAV genome during terminal resolution (Fig. 4). An additional higher-molecular-weight species also was seen in this experiment. Presumably, the higher-molecular-weight band was due to the small amount of Rep78 present in the crude cytoplasmic extract or was due to an incomplete digestion of the protein-DNA complex by DNase I and micrococcal nuclease. Finally, an in vitro reaction in which an adenovirus-infected cell extract was used did not result in the incorporation of significant radioactivity into the protein (Fig. 4, left lane).

To determine whether the protein attached to the 5′ end of the *trs* site during the *trs* endonuclease reaction is the same as the protein which attached to the 5′ ends of AAV DNA during the in vitro resolution reaction, the two reactions were compared by using radiolabeled NE substrate (Fig. 5). In the case of the *trs* endonuclease reaction, a partially purified preparation of Rep68 was used [Rep68 (I) (32)]. This fraction was free of Rep78, Rep52, and Rep40 (Fig. 5, right panel) as well as the AAV capsid proteins (31). Both sets of reaction products were digested with nucleases as before

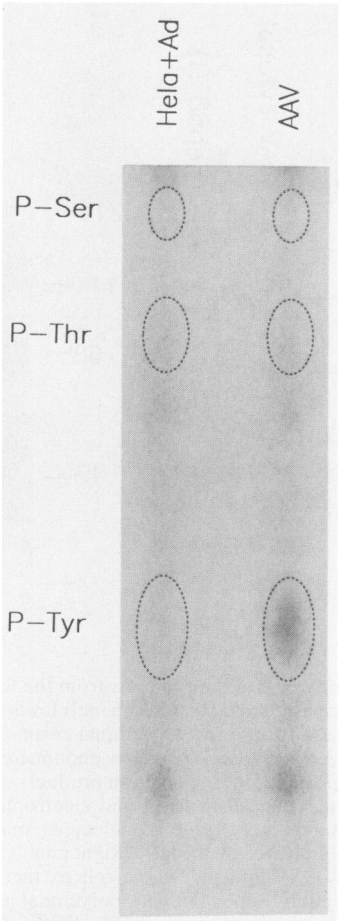

FIG. 6. Phosphoamino acid analysis. Radiolabeled NE substrate was added to resolution reactions containing either adenovirus- (lane Hela+Ad) or adenovirus-plus-AAV (lane AAV) infected cellular extracts. The products were digested with DNase I, micrococcal nuclease, and pronase E. The reaction mixtures were then hydrolyzed in 6 N HCl at 110°C, lyophilized, dissolved in 10 mM Tris (pH 8.0) containing the *O*-phosphoamino acid markers, spotted onto Whatman 3MM paper, and electrophoresed at pH 3.5. The *O*-phosphoserine (P-Ser), *O*-phosphothreonine (P-Thr), and *O*-phosphotyrosine (P-Tyr) markers were visualized with ninhydrin, and their mobilities are indicated by the dotted ovals. The ^{32}P-labeled amino acid was visualized by autoradiography.

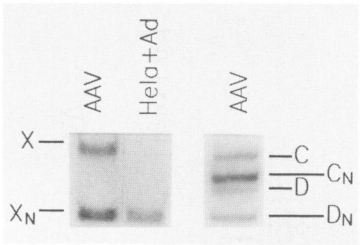

FIG. 7. Efficiency of the resolution reaction. (Left panel) Radiolabeled NE substrate was added to an in vitro resolution reaction containing either adenovirus- (lane Hela+Ad) or adenovirus-plus-AAV- (lane AAV) infected HeLa cell extracts. The products were digested with proteinase K, extracted with phenol-chloroform-isoamyl alcohol, and precipitated with ethanol. The products were then dissolved in water, digested with *Pst*I and *Xba*I, and electrophoresed on a 4% nondenaturing polyacrylamide gel. X and X_N are the resolved and unresolved terminal *Xba*I fragments, respectively. (Right panel) The products of a resolution reaction in which an adenovirus-plus-AAV-infected cellular extract, nonradioactive NE substrate, and unlabeled deoxynucleoside triphosphates were used. The reaction products were prepared as described above, digested with *Pst*I, electrophoresed on a 4% nondenaturing polyacrylamide gel, transfered to a nylon membrane, and hybridized to ^{32}P-labeled AAV DNA. C and D are the resolved terminal *Pst*I C and D fragments, respectively; C_N and D_N are the unresolved terminal *Pst*I fragments. See Fig. 3c for the locations of these fragments.

and separated on a 10% SDS-polyacrylamide gel. In both cases, a protein band of approximately 65 kDa was labeled (Fig. 5, left panel). We interpret these results to mean that Rep68 becomes covalently attached to the template strand of the AAV genome during both the in vitro resolution and the *trs* endonuclease reactions.

Attachment of Rep to DNA via tyrosine. To determine which amino acid in Rep68 was covalently attached to the ends of resolved AAV DNA, the products of the in vitro resolution reaction were hydrolyzed in acid to generate the *O*-phosphoamino acid residues. The hydrolyzed products were separated by paper electrophoresis in one dimension at pH 3.5, and the *O*-phosphoamino acid markers were visualized with ninhydrin (29). Radioactivity comigrated with the *O*-phosphotyrosine marker when the adenovirus- and AAV-infected extract was used, and no radioactivity comigrated with *O*-phosphotyrosine when an extract infected with adenovirus alone was used (Fig. 6). This result supported our previous suggestion that the DNA-protein bond involves a tyrosine residue because of its resistance to cleavage by piperidine (66). In addition, no radioactivity comigrated with the *O*-phosphothreonine or *O*-phosphoserine markers when either adenovirus-infected or adenovirus-plus-AAV-infected extracts were used. The radioactive spots seen running slower than the *O*-phosphotyrosine marker comigrated with thymidine 3′-monophosphate and 2′-deoxyguanosine 3′-monophosphate (Fig. 6). We concluded that Rep68 was attached to the 5′ ends of AAV DNA via a tyrosine residue. Furthermore, because the NE substrate DNA had been radiolabeled by the incorporation of [α-^{32}P]TMP, we concluded that Rep68 was attached to the α-phosphate of a thymidine residue in the AAV genome. Because radiolabeled NE substrate was not synthesized with other [α-^{32}P]deoxynucleoside triphosphates, we have not determined whether Rep68 attaches exclusively to thymidine residues or is capable of covalently attaching to other nucleotides. Since the sequence found at the 5′ ends of virion DNA is heterogeneous (23) and the size of the terminal fragment of in vitro resolved products is heterogeneous (66), it is possible that Rep is capable of nicking and attaching to other nucleotides.

Efficiency of in vitro resolution. In our previous studies, we could not measure the efficiency of in vitro terminal resolution accurately using unlabeled NE substrate and radioactive deoxyribonucleoside triphosphates (66). This was because radioactivity was incorporated almost exclusively into resolved AAV termini. Unresolved AAV termini and internal AAV sequences incorporated significantly smaller amounts of radioactivity by what appeared to be a different mechanism. To determine what percentage of the input NE substrate is resolved, radiolabeled NE DNA was used as the substrate in the in vitro resolution reactions with adenovirus-infected or adenovirus-plus-AAV-infected cellular extracts. Approximately 40% of the covalently joined AAV chromosome ends (X_N) were converted to the resolved open duplex form (X) under standard reaction conditions (Fig. 7, left

TABLE 1. Requirements for in vitro resolution[a]

Reaction mixture alteration	% Relative activity	Extract source[b]
None (complete)	100	Ad+AAV
+Aphidicolin (10 μg/ml)	5	Ad+AAV
+BuPdGTP (40 nM)	110	Ad+AAV
+BuAdATP (20 nM)	110	Ad+AAV
+BuPdGTP (120 μM)	50	Ad+AAV
+BuAdATP (70 μM)	50	Ad+AAV
+α-Amanitin (10 μg/ml)	100	Ad+AAV
+Camptothecin (200 μM)	100	Ad+AAV
+VM26 (200 μM)	100	Ad+AAV
+Dimethyl sulfoxide	100	Ad+AAV
−CTP, GTP, and UTP	100	Ad+AAV
−ATP	0	Ad+AAV
−Rep68	0	Adenovirus
+Rep68[c]	100	Adenovirus
−Rep68	0	Uninfected
+Rep68[c]	100	Uninfected

[a] The amount of resolved terminal *Xba*I product synthesized in the complete standard in vitro resolution reaction (see Materials and Methods and Fig. 7) was quantitated by densitometry and arbitrarily set to 100%. The amount of product produced under the other reaction conditions was also quantitated by densitometry and is presented as the percent activity seen in the complete standard reaction. Unlabeled NE substrate and [α-^{32}P]dCTP were used in all of the reactions.

[b] Cytoplasmic crude extracts were made from adenovirus-plus-AAV-infected HeLa cells (Ad+AAV), from adenovirus-infected HeLa cells (Adenovirus) or from uninfected HeLa cells (Uninfected).

[c] Rep68 (I) (5 U) was added to the standard reaction mixture as described in Materials and Methods.

panel, lane AAV). This was confirmed by hybridization analysis of the products of in vitro reactions in which unlabeled NE substrate deoxyribonucleoside triphosphates were used and in which the products were subsequently cut with *Pst*I (Fig. 7, right panel).

Requirements for terminal resolution. A series of experiments was carried out to identify some of the protein and cofactor requirements for the in vitro terminal resolution reaction (Table 1). Aphidicolin at a concentration of 10 μg/ml inhibited resolution more than 90%. This suggested that either one or both of the cellular DNA polymerases α and δ were primarily responsible for the DNA synthesis that occurs in the in vitro resolution reaction (12, 30, 38, 41, 67). The remaining DNA synthesis could be due to cellular DNA polymerases β or γ, which are both resistant to aphidicolin (41, 67). To distinguish between polymerase α and δ, we used the nucleotide analogs *p-n*-butylphenyl dGTP (BuPdGTP) and *p-n*-butylanilino dATP (BuAdATP) in the in vitro resolution reaction (39, 67). A titration of BuPdGTP and BuAdATP indicated that 50% inhibition of terminal resolution occurred with 120 μM BuPdGTP and 70 μM BuAdATP (Table 1). This suggested that polymerase δ was primarily responsible for the DNA synthesis that occurred during the in vitro resolution reaction. Essentially no inhibition was seen at concentrations of BuPdGTP (40 nM) and BuAdATP (20 nM) that were expected to inhibit polymerase α (Table 1) (39).

When α-amanitin was added to the reaction at 10 μg/ml, no effect was detected (Table 1), suggesting that the host RNA polymerase II was not required for the reaction (56). No inhibition was observed when the topoisomerase I inhibitor camptothecin and the topoisomerase II inhibitor VM26 were added (79). Dimethyl sulfoxide, the solvent used to dissolve aphidicolin, camptothecin, and VM26, also had no effect on the reaction (Table 1). The omission of ATP, phosphocreatine, and phosphocreatine kinase inhibited the reaction completely. Omission of the other ribonucleotides had no effect (Table 1).

Extracts made from uninfected cells or cells infected only with adenovirus were unable to resolve the covalently joined ends of NE substrate (66). To determine whether the AAV-encoded Rep protein was the only viral protein required by cellular extracts for resolution, purified Rep68 protein was added to in vitro resolution reaction mixtures which contained extracts from uninfected or adenovirus-infected HeLa cells (Table 1). In both cases, resolution of the covalently joined ends was observed. We concluded that the AAV Rep68 protein was the only protein required to make crude cellular extracts competent for carrying out the terminal resolution reaction.

DISCUSSION

Covalent attachment of p5 Rep proteins. In previous studies, we observed that a protein became attached to the 5′ ends of the AAV genome during the resolution of covalently joined AAV termini in vitro and during the in vitro *trs* endonuclease reaction (32, 66). This attachment was insensitive to proteases, phenol, alkali, heat, 8 M urea, and SDS (32, 66; Im and Muzyczka, unpublished data). In this report, the identity of this protein and the nature of the linkage between the protein and the 5′ ends of the AAV genome were investigated. To approach these problems, radiolabeled NE DNA was used as a substrate to show that radioactivity could be transferred from DNA to the terminal protein. When the protein-DNA complex generated in the in vitro resolution and *trs* endonuclease reactions was digested with endonucleases, a radioactive protein of approximately 65 kDa was observed (Fig. 4 and 5, left panel). This protein was labeled only if an adenovirus-plus-AAV-infected extract or a partially purified preparation of Rep68 was used. Furthermore, the labeled protein migrated with a mobility identical to that of Rep68. Finally, we have shown elsewhere (32) that when Rep68 was purified to apparent homogeneity, it retained the ability to form a protein-DNA complex with nicked AAV termini. Taken together, these results indicated that the product of both the *trs* endonuclease reaction and the terminal resolution reaction was a covalent protein-DNA complex between the nicked or resolved terminal AAV sequence and the Rep68 protein. In the experiments presented here, the cytoplasmic extract and the purified Rep68 fraction contained little if any Rep78. However, in experiments that will be presented elsewhere (Im and Muzycka, unpublished data), we have demonstrated that Rep78 has the same biochemical activities as Rep68, including the ability to form a covalent protein-DNA product during the *trs* endonuclease reaction. In addition, we have shown previously (32) that both Rep68 and Rep78 can be separated into two molecular weight species during electrophoresis on SDS-acrylamide gels (Fig. 5, right panel). We were unable, however, to determine whether there was any difference between the two forms of Rep68 in their ability to form a covalent linkage.

Neither of the two p19 Rep proteins, Rep52 and Rep40, contained detectable levels of radioactivity after incubation of crude AAV-infected extracts in the terminal resolution assay. This was true in spite of the fact that the extracts contained substantial amounts of the p19 Rep proteins. The inability of the p19 Rep proteins to form a covalent protein-DNA complex suggested that the N-terminal sequences of Rep78 and Rep68, which are present only in the p5 Rep

proteins, are essential either for binding or nicking the AAV terminal hairpin. It was also possible that the tyrosine residue required for the covalent linkage is absent in the p19 Rep proteins. Furthermore, our experiments demonstrated clearly that neither Rep52 nor Rep40 were essential for any aspect of the terminal resolution reaction, which is consistent with the results of Chejanovsky and Carter (14). Purified Rep68, when added to an uninfected HeLa cell extract, was sufficient for resolution.

Functional significance of the covalent tyrosine linkage. Our results demonstrate that the linkage to Rep68 is between a tyrosine residue and the 5′ end of resolved AAV DNA. This result is consistent with our previous observation that the DNA-protein bond is resistant to cleavage with piperidine (66) and with reports from other laboratories which show that a number of parvoviruses have terminal proteins bound by alkali stable linkages to the 5′ ends of their genomes (17, 49, 50, 55, 77). The experiments presented here and previously (32, 66) demonstrate that the covalent attachment of the p5 Rep proteins is the result of a site-specific nick which occurs at the *trs* site. Since all parvoviruses are believed to undergo a terminal resolution or hairpin transfer step (3, 5, 16), it is likely that all parvoviruses also produce a covalent protein-DNA complex during DNA replication. Thus far, about half of the members of the parvovirus family (minute virus of mice [MVM], H1, LuIII, and Kilham rat virus) have been shown to have stable protein-DNA linkages in vivo; the remainder (Aleutian disease virus, bovine parvovirus, human B19 parvovirus, and the insect parvoviruses) are similar to AAV in that a protein-DNA linkage has not been detected during viral infection (17, 49, 50, 55, 77).

In the case of MVM, the covalently linked protein is the virus-coded nonstructural protein NS1 (17). Mutations within the MVM NS1 protein have essentially the same phenotype as mutations within the AAV Rep protein (16), and there is substantial similarity between the amino acid sequences of the two proteins (4). The linkage between the NS1 protein and MVM DNA is quite stable and persists even after the viral DNA has been packaged. It is believed to be removed by an endonuclease which removes both the protein and a short duplicated fragment of the MVM terminal sequence that is present in replicative-form DNA (18). In contrast, there is no evidence that a similar mechanism plays a role in AAV DNA replication or virus maturation. We have not found a duplication at the ends of AAV replicative-form molecules in vivo, and the terminal sequence that we find attached to the Rep protein in vitro is essentially identical to the one which is packaged in vivo (23, 32, 66). Furthermore, the AAV DNA which is packaged does not have a covalently attached protein. The comparison between the AAV Rep and MVM NS1 linkages suggests two points. First, the fact that a covalent protein-DNA complex has not been found in previous studies of AAV-infected cells means that there must be some efficient way in vivo of breaking the covalent linkage. The second point is that, unlike MVM, the mechanism of removing the protein from the ends of AAV DNA probably involves breaking the tyrosine phosphate bond directly. If this is true, it would mean that the parvoviruses have evolved at least two mechanisms for removing the 5′-end protein that is attached during the terminal resolution or hairpin transfer step.

We are not certain why it is necessary to form a covalent attachment during terminal resolution; however, the function of covalent attachment in DNA replication and the question of how the covalently attached Rep protein is removed are probably related. We can imagine several possible reasons for the covalent attachment. One function of the covalent linkage may be to prevent DNA ligase from resealing the nick at the *trs* site before resolution can occur. This might be necessary if the assembly of a DNA polymerase complex at the terminal repeat is a rate-limiting step in terminal resolution. Another possibility is that the terminal protein is required for viral DNA packaging. Conceivably, the terminal protein promotes an interaction with capsid proteins and targets the associated DNA strand for packaging. In this regard, it is worth noting that the AAV packaging signal is located within the AAV terminal repeat (46, 61) and that the capsid proteins of two other parvoviruses, Aleutian disease virus and bovine parvovirus (37, 76), are capable of binding to their respective terminal repeats. Furthermore, in the case of LuIII, it has been found that the removal of the terminal protein occurs concomitantly with viral packaging (49, 50). A third possibility stems from our demonstration that Rep68 has an associated DNA helicase activity (32). It is possible that the covalent linkage is a way of positioning the Rep helicase activity so that it is available for unwinding the terminal repeat.

None of the ideas discussed above provide a mechanism for breaking the tyrosine phosphate linkage. Thus far, we have not found in vitro conditions which promote unlinking of the Rep protein from AAV DNA. Conceivably, removal of the Rep protein could be catalyzed by a cellular activity. For instance, the removal of the poliovirus terminal protein, Vpg, from the 5′ ends of the poliovirus genome has been shown to occur in the presence of an enzyme found in uninfected HeLa cells (1, 2). Whether this activity is responsible for removing Rep protein from the ends of the AAV genome remains to be investigated. It is also possible that the Rep protein is a nicking-joining enzyme which has a function during AAV DNA replication similar to that of the gene A protein of ϕX174 (10, 22, 35, 54, 57, 63, 75). A site-specific nicking-joining activity has been postulated to be required by some autonomous parvoviruses during DNA replication (3, 15, 16) and by analogy may be required during AAV DNA synthesis. As yet, however, we have not found reaction conditions or substrates which promote the transfer of the terminal protein to a new AAV end or the ligation of a covalently attached 5′ DNA end to a free 3′ OH. It is worth noting also that the nicking activity of Rep68 was not inhibited by the topoisomerase inhibitors that were used here and that we have not detected topoisomerase activity in purified Rep68 fractions as judged by their ability to relax supercoiled form I plasmid DNA (D.-S. Im and N. Muzyczka, unpublished data).

Cellular enzymes involved in terminal resolution. In virtually every respect, the results of the inhibitor studies of the in vitro resolution reaction are consistent with in vivo studies of AAV DNA replication. First, several lines of evidence have suggested that AAV DNA replication relies on a cellular DNA polymerase. A temperature-sensitive mutation in the adenovirus DNA polymerase gene supports AAV DNA replication as efficiently as does wild-type adenovirus (69; S. K. McLaughlin and N. Muzyczka, unpublished data). Furthermore, members of the herpesvirus family also can supply helper functions for AAV as efficiently as adenovirus (11, 47), and several laboratories have demonstrated that under some conditions, AAV DNA replication can occur in the absence of a helper virus coinfection (64, 78). Finally, AAV DNA replication in vivo is almost completely inhibited by aphidicolin (McLaughlin and Muzyczka, unpublished data), as is terminal resolution in vitro. This occurs at concentrations of aphidicolin which are effective

for inhibiting cellular DNA replication (19, 30). Taken together, these observations suggest that both in vivo and in vitro, AAV DNA synthesis relies primarily on an aphidicolin-sensitive cellular DNA polymerase, either polymerase α or δ. We note that there was a small amount of residual activity in vitro in the presence of aphidicolin. A similar observation has been made in vivo (McLaughlin and Muzyczka, unpublished data). We are not certain which DNA polymerase is responsible, but this residual DNA synthesis may be related to the DNA synthesis of partially purified AAV replicative-form complexes which was observed by Handa and Carter (27).

The DNA synthesis which occurs during terminal resolution is a form of leading-strand synthesis, and it was not expected to require RNA priming (Fig. 1). It is not surprising, therefore, that with the exception of ATP, ribonucleoside triphosphates are not essential cofactors for terminal resolution. ATP was shown previously to be required for the Rep68 site-specific endonuclease and DNA helicase activities (32). Finally, recent studies of simian virus 40 and cellular DNA replication have led to the suggestion that leading- and lagging-strand DNA synthesis are due to different cellular DNA polymerases, polymerases δ and α, respectively (12, 19–21, 39, 51–53). Our observation that terminal resolution is essentially insensitive to the nucleotide analogs BuPdGTP and BuAdATP suggests that the DNA synthesis during terminal resolution is due to polymerase δ and is consistent with the idea that polymerase δ is primarily responsible for leading-strand synthesis (20, 52). We did not determine whether the polymerase δ that is involved in terminal resolution is dependent on proliferating cell nuclear antigen (8, 9, 24, 51, 53, 71, 72), but we expect that further studies of in vitro AAV DNA replication will resolve this question. Such studies also should be generally useful for identifying those cellular enzymes which are exclusively involved in leading-strand DNA synthesis.

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