Adeno-associated virus type 2 Rep endonuclease activity is necessary for both viral DNA replication and site-specific integration of the viral genome into human chromosome 19. The biochemical activities required for site-specific endonuclease activity (namely specific DNA binding and transesterification activity) have been mapped to the amino-terminal domain of the AAV2 Rep protein. The amino-terminal 208 amino acids are alone sufficient for site-specific endonuclease activity, and nicking by this domain is metal-dependent. To identify this metal-binding site, we have employed a cysteine mutagenesis approach that targets conserved acidic amino acids. By using this technique, we provide functional biochemical data supporting a role for glutamate 83 in the coordination of metal ions in the context of Rep endonuclease activity. In addition, our biochemical data suggest that glutamate 164, although not involved in the coordination of metal ions, is closely associated with the active site. Thus, in lieu of a crystal structure for the AAV type 2 amino-terminal domain, our data corroborate the recently published structural studies of the AAV type 5 endonuclease and suggest that although the two enzymes are not highly conserved with respect to the AAV family, their active sites are highly conserved.

AAV2 is a human parvovirus (for review see Ref. 1) that has the unique ability to integrate its genome site-specifically into a defined locus of human chromosome 19q13.4, known as AAVS1 (2–9). This event occurs under conditions that do not favor productive replication (i.e. absence of helper virus coinfection), thereby establishing a latent infection (10–12). Although intermediate steps in the integration process remain undefined, its initiation is thought to parallel events that occur at the viral origin of replication during a productive infection (13, 14).

The AAV2 minimal origin of DNA replication consists of two motifs, the RBS and TRS (6, 15, 16). During viral DNA replication, the virally encoded Rep protein binds to the RBS. This event is followed by site- and strand-specific endonuclease activity directed at the TRS. Through this activity, Rep generates a 3’-hydroxyl group to allow for unidirectional DNA replication of its hairpinned ends (17). The RBS and TRS motifs are also present within AAVS1, and together with Rep have been shown to be both necessary and sufficient for targeted AAV2 integration at this site (6). Current models suggest that Rep interacts with the AAVS1 motifs in a manner that is similar to its interaction with the viral origin of DNA replication thereby targeting the AAV2 genome for integration at this site (8, 13).

The amino-terminal domain of AAV Rep has been shown to contain all of the residues necessary for site-specific DNA binding and endonuclease activity (14, 18, 19). This domain includes an active tyrosine residue, Tyr-156, which is conserved in all parvovirus nonstructural Rep proteins. This residue is responsible for the covalent attachment of Rep during transesterification to the nicked DNA strand through a 5’-phosphotyrosyl linkage to a thymidine residue at the TRS (20, 21). As expected, mutation of this active site tyrosine results in the abrogation of TRS nicking activity (19, 22). Both Davis et al. (19) and our laboratory (14) have demonstrated that Rep variants consisting of the amino-terminal 200 or 208 amino acids, respectively, retained specific endonuclease activity. Sequence comparison between related parvoviruses reveals that Asn-208 is within a region that is not conserved, suggesting that it may serve as a linker between the endonuclease and highly conserved helicase domains. Taken together, these observations suggest that the relevant boundary with respect to endonuclease function lies close to asparaginase 208 and thus defines the endonuclease domain.

A prerequisite to nicking by Rep is that the TRS is single-stranded, which in vitro is accomplished via ATP- and Mg²⁺-dependent Rep helicase activity (23). On a single-stranded TRS substrate, helicase activity is no longer a prerequisite for nicking activity. Therefore, endonuclease activity itself is ATP-independent. However, the presence of a divalent metal cofactor is absolutely necessary. Our previous data using the truncation variant Rep78N208 showed that the amino-terminal domain is able to nick the TRS in a metal-dependent manner. These observations support that, in addition to the putative metal-binding site for helicase activity and the carboxyl-terminal zinc fingers (24), there exists an independent metal-binding site dedicated to endonuclease activity within the amino-terminal domain.

The existence of an amino-terminal metal-binding site for Rep has recently been confirmed by the crystal structure of the AAV type 5 Rep endonuclease domain (25). Hickman et al. (25) have demonstrated that two histidines and a glutamate residue act to coordinate the divalent metal ion. Interestingly, type 5 and type 2 appear to be among the most disparate serotypes among the AAV family members (26). However, using a conditional mutagenesis approach specific for acidic residues, we demonstrate a metal binding role for the same glutamate residue act to coordinate the divalent metal ion. Interestingly, type 5 and type 2 appear to be among the most disparate serotypes among the AAV family members (26). However, using a conditional mutagenesis approach specific for acidic residues, we demonstrate a metal binding role for the same glutamate residue.
that Glu-164 is likely to be in close proximity to the active site. Taken together, the coordination of the structural data for AAV5 Rep and the functional data described here serve to provide an accurate picture of the AAV2 Rep endonuclease domain.

**EXPERIMENTAL PROCEDURES**

**Cloning of Mutant Rep Expression Constructs**—All mutant Rep proteins were generated using pHisRep68/15b, which contains the AAV2 rep68 gene in a pET15b vector (Novagen). Site-directed mutagenesis for mutants D14C and E83C were generated via overlapping PCR using NdeI and SacI for cloning back into pHisRep68. Mutants E68C, E126C, E136C, E141C, E146C, E152C, E163C, E176C, E184C, E201C, E86A, and E164A were generated using the QuikChange Site-directed mutagenesis kit (Stratagene). Double mutants E83C/Y156F, and E83C/K340H were generated by subcloning sequences carrying one mutation into vectors carrying the complementary mutation. In all cases, sequences generated by PCR were confirmed by sequencing.

**Protein Isolation**—All recombinant proteins were isolated via nickel-nitrilotriacetic acid column chromatography as described previously (14). 50-ml cultures of BL21(DE3)pLysS cells were prepared and induced as per the manufacturer’s instructions (Novagen), and induced bacterial pellets were frozen at −80 °C until processed. Column chromatography, using 1-ml Hi-Trap Chelating columns (Amersham Biosciences) was performed as per the manufacturer’s instructions using the His-Bind Buffer kit (Novagen). Proteins were eluted in 300 mM imidazole and desalted over PD-10 columns (Amersham Biosciences) into a buffer containing 25 mM Tris HCl (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 20% glycerol. Proteins were aliquoted and stored at −80 °C until use. Protein concentrations were estimated by Coomassie Blue staining after SDS-PAGE and were adjusted to 100 ng of Rep. Reactions were incubated for 45 min at 37 °C and terminated by the addition of 1 μl of 0.5 μM EDTA and 3 μl of loading buffer. Reactions were run on 6% native polyacrylamide gels in 1× TBE and treated as per the EMSA and helicase assay.

**RESULTS**

**Identification of Candidate Residues for Metal Binding**—Cysteine replacement of acidic residues has been used previously to identify the metal-binding sites of several endonucleases of the “DDE” motif-containing family. These include TnsA, a subunit of the Tn7 transposase (27), the Tn10 transposase (28), and most recently, the RAG1 endonuclease which facilitates V(D)J recombination of the immunoglobulin locus (29, 30). This method takes advantage of the different side chain chemistries between acidic amino acids, aspartate and glutamate, and cysteine with respect to their ability to interact with divalent metal ions Mg2+ and Mn2+ (31). The oxygen of aspartate and glutamate acts as a good ligand for both metal ions, whereas the sulfur of cysteine is only able to interact efficiently with Mn2+. Accordingly, a cysteine substitution of an acidic amino acid that results in a change in the divalent metal ion requirement for catalysis strongly implicates that residue in metal binding.

A multiple sequence alignment of the AAV2 Rep endonuclease domain (amino acids 1–208) (14) with the nonstructural proteins from different parvoviruses was performed. This analysis resulted in the identification of several candidate metal-binding residues (ClustalW alignment, Blosum similarity matrix, open gap penalty = 10, extend gap penalty = 0.1). A total of 3 aspartate and 13 glutamate residues were highly conserved (Fig. 1A). Of these, residues Glu-6, Asp-14, Asp-16, Glu-57, Glu-75, Glu-96, Glu-114, Glu-125, Glu-173, Glu-184, and Glu-201 had no effect on nicking activity (32) and were controls for this study. All variants used in this study were cloned into bacterial expression vectors that place a His6 tag at the amino terminus of the protein. Recombinant proteins were isolated by nickel-nitrilotriacetic acid affinity chromatography in comparable quantities and at greater than 95% purity (Fig. 1B). It should be noted that variant E6C required a lower induction temperature (18 °C) in order for the protein to be recovered in the soluble fraction.

**Nicking Activity of Mutant Rep Proteins**—In order to determine whether or not the cysteine mutants displayed conditional activity in the presence of either Mg2+ or Mn2+, TRS endonuclease assays were performed using two related substrates (Fig. 2). Both substrates contain the AAV2 minimal origin of DNA replication and differ in whether or not the TRS region is single-stranded (ssTRS, Fig. 2A) or double-stranded (dsTRS, Fig. 2B). Wild type Rep68 protein retains nicking activity in both Mg2+ and Mn2+ regardless of the state of the TRS; although consistent with previous studies, the wild type enzyme clearly favors Mn2+ on the ssTRS substrate (22), lanes 2–4, top and bottom) (14, 22). Mn2+ enhancement of nicking is not as pronounced when the dsTRS is used (Fig. 2B, lanes 5–7).
some extent in the presence of Mn$^{2+}$ (Fig. 2A). Alanine mutagenesis (32) has been implicated in endonuclease activity previously by charge-to-phenotype in that they were active in both divalent metal ions (data not shown). This supports the notion that although Asp/Glu to Cys substitutions are not conservative, they are tolerated by the enzyme. E6C is an exception, in that it showed no activity in our assays. As noted above, it was not possible to isolate this mutant under the same conditions as the others, suggesting that this mutation had a more global effect on protein integrity.

Rescue of E83C Nicking Activity on the dsTRS Substrate—

The decreased ability of E83C nicking activity to be rescued in Mn$^{2+}$ on the dsTRS compared with the ssTRS could be the result of competition between the putative metal-binding site of the helicase domain and that of the endonuclease domain on the same metal cofactor pool. This prospect is magnified because it has been demonstrated that Rep helicase activity is not efficiently supported in Mn$^{2+}$ at the same concentrations as in Mg$^{2+}$ (21, 33). To overcome this possibility, nicking assays using E83C on the dsTRS substrate were performed such that both Mg$^{2+}$ and Mn$^{2+}$ were titrated into the same reaction (Fig. 3). Although the wild type enzyme was active over a broad range of total Me$^{2+}$ concentrations, nicking by E83C was only observed when Mn$^{2+}$ was present in the reaction (Fig. 3, lanes 13–19). Strong nicking activity by E83C, comparable with the wild type enzyme, was restored at an Mg$^{2+}$ to Mn$^{2+}$ ratio of 1:1 (Fig. 3, lanes 13 and 17).

The lack of nicking activity in Mg$^{2+}$ by E83C and E164C could also be attributed to a decreased affinity for the metal due to the substitution. In order to rule out this possibility, experiments were performed in which each divalent metal cofactor was titrated into the reaction. This approach assumes that a lower affinity for the divalent cation could be overcome by an increase in its concentration in the reaction. As Fig. 4 shows, an increase in the concentration of Mg$^{2+}$ on either the ssTRS (A) or dsTRS (B) substrate did not result in recovery of nicking activity for either variant, in support of our hypothesis that the activity of the cysteine substitution mutants is specifically dependent on Mn$^{2+}$. The origin of the higher migrating products in the presence of Mg$^{2+}$ on the dsTRS substrate (Fig. 4B, lanes 6, 13, and 19) is unknown. However, it is possible that they may be the result of a conformational change in the DNA substrate that occurs at high magnesium concentrations that promote nonspecific nicking by Rep. A control mutant, E83C/Y156F, did not possess any nicking activity, thus making the possibility of a copurifying, nonspecific nuclease unlikely (data not shown).

Conditional Phenotype Is Specific to the Cys-Mn$^{2+}$ Interaction—

In order to verify that the conditional phenotype displayed by E83C and the partially conditional phenotype of E164C was specific for the presence of cysteine at these positions, single alanine substitutions of these residues were made. As expected, mutant E83A nicking activity was shown to be severely impaired (Fig. 5, lane 14), suggesting that Glu-83 does not act alone in metal binding. The generation of E164A allowed us to determine the relative contribution of this residue to nicking when compared with wild type Rep68 and Glu-83 variants. Because the nicking phenotype of E164C was not fully conditional, we hypothesized that this residue may not play a direct role in metal binding but may perhaps act to maintain the integrity of the metal-binding

lanes 2–4, top and bottom). Cysteine mutants D14C, D16C, and D42C follow a wild type-like pattern of nicking in that they retain activity on both substrates in the presence of either divalent metal ion. E83C, however, clearly displays a conditional phenotype. Although nicking is barely detectable in Mg$^{2+}$ (Fig. 2, A and B, top, lanes 17–19), it is recoverable to some extent in the presence of Mn$^{2+}$ on the dsTRS substrate (Fig. 2B, bottom, lanes 17–19) and to significant levels on the ssTRS substrate (Fig. 2A, bottom, lanes 17–19). This switch in divalent metal ion requirement compared with the wild type enzyme strongly implicates this residue in coordination of the metal cation. Although not as remarkable as the conditional phenotype observed for E83C, E164C nicking activity is also suppressed in Mg$^{2+}$ but rescued in Mn$^{2+}$ (Fig. 2, A and B, top and bottom, lanes 20–22). Nicking was not observed when a divalent metal cation was omitted from the reaction or when an endonuclease negative variant, Y156F, was used (data not shown).

The results from the experiment in Fig. 2 are summarized in Table I. The total nicking activity of each of the variants was quantified relative to that of wild type Rep68 on each substrate and metal ion. Overall, that the nicking activity of E83C and E164C is suppressed in Mg$^{2+}$ and active in Mn$^{2+}$ is apparent, although the extent of rescue is more pronounced on the ssTRS substrate. Mg$^{2+}$ is unable to efficiently support nicking by E83C or E164C on the ssTRS (0 and 11% of wild type, respectively). For both variants, nicking is restored in the presence of Mn$^{2+}$ to approximately half of wild type Rep68 levels (55% for E83C and 66% for E164C). The control variants, Glu-36, Glu-49, Glu-57, Glu-75, Glu-96, Glu-114, Glu-125, Glu-173, Glu-184, and Glu-201 were assayed in the same manner as described in Fig. 2 and, as predicted, possessed a wild type phenotype in that they were active in both divalent metal ions (data not shown). This supports the notion that although Asp/Glu to Cys substitutions are not conservative, they are tolerated by the enzyme.
pocket within the enzyme. This hypothesis is based on the position of Glu-164 and its relative proximity to the active tyrosine residue at position 156 with respect to the amino acid sequence. However, E164A activity was only slightly affected when compared with the wild type enzyme, indicating that Glu-164 does not play a significant role, if any, in metal interactions. Other divalent metal cations were tested for their ability to support nicking by the conditional mutants. Zn\(^{2+}\) and Ca\(^{2+}\)-supplemented buffers were also used for the nicking assay, respectively. Wild type Rep showed very weak activity in Ca\(^{2+}\), in addition to the stronger activity typically observed in

### TABLE I

| Substrate nicking by cysteine mutants in the presence of Mg\(^{2+}\) or Mn\(^{2+}\) divalent metal cofactor. |
|--------------------------------------------------|
| Nicking values are relative. Nicking of wild type Rep68 was set to 1.  |
| Values represent the average of triplicate assays.  |
| ssTRS       | dsTRS       |
|-------------|-------------|
| Wild type   | ~1          | ~1          |
| E6C         | 0.00        | 0.05        |
| D14C        | 0.88        | 0.77        |
| D16C        | 0.89        | 0.65        |
| D42C        | 1.00        | 0.75        |
| E83C        | 0.00        | 0.00        |
| E164C       | 0.11        | 0.04        |

Fig. 2. E83C and E164C display a conditional nicking phenotype. Endonuclease assays were performed on linear AAV origin DNA substrates that contained either a single-stranded TRS (A, ssTRS) or a substrate in which the TRS was double-stranded (B, dsTRS). Reactions were supplemented with either 5 mM MgCl\(_2\) (top) or 5 mM MnCl\(_2\) (bottom). Products of the nicking reaction were resolved on a 15% polyacrylamide, 50% urea (w/v) gel. Under each condition, lane 1 represents the substrate incubated in the presence of the indicated metal ion, but in the absence of enzyme; S, substrate.

Fig. 3. Optimal rescue of nicking by E83C on the dsTRS substrate requires both Mg\(^{2+}\) and Mn\(^{2+}\). Magnesium and manganese ion concentrations were counter-titrated against each other in standard nicking assays. In each case, the concentration of one ion was fixed at 5 mM, and the other ion was titrated at 5, 10, and 15 mM, respectively (lanes 4–6, 8–10, 13–15, and 17–29). Lanes 2 and 11 contain reactions incubated without divalent metal cation, whereas lanes 3 and 12 represent reaction in which only one ion was present. Lane 1 represents kinase-labeled marker oligonucleotides corresponding to the expected products of the nicking reaction incubated under the same buffer conditions. S, substrate; P, product.

Fig. 4. The conditional phenotype of E83C and E164C cannot be overcome by increasing Mg\(^{2+}\) concentrations. Nicking activity of wild type Rep68, E83C, and E164C in the presence of increasing amounts of either MgCl\(_2\) (Mg) or MnCl\(_2\) (Mn) as indicated, on the ssTRS (A) or dsTRS (B) substrate. Lanes 3 and 7 contain 3.125 mM of the indicated metal cofactor; lanes 4, 8, 12, 14, 18, and 20 contain 6.25 mM metal cofactor; lanes 5, 9, 13, 15, 19, and 21 contain 12.5 mM metal cofactor; lanes 6, 10, 16, and 22 contain 25 mM metal cofactor. Lane 1 does not contain enzyme or metal cofactor. S, substrate; P, product of nicking at the TRS.
Mg$^{2+}$ and Mn$^{2+}$. However, nicking by the cysteine-substituted enzymes was only efficiently supported by Mn$^{2+}$, as expected. 

Zn$^{2+}$ was unable to support nicking by any of the proteins tested, as reported previously (21, 34–36).

**Glu-83 and Glu-164 Do Not Play a Role in Origin DNA Binding or Helicase Activity**—Because origin binding and helicase-mediated extrusion of the TRS (on the dsTRS substrate) are prerequisites to endonuclease activity, it is possible that the conditional phenotype observed for E83C and the partially conditional phenotype for E164C are not specific to endonuclease activity per se but have their effect on the steps prior to transsterification at the TRS. To rule out this possibility, E83C and E164C were tested for the origin binding and helicase activities in separate assays under conditions that did not favor their nicking activity, i.e. in buffers lacking Mn$^{2+}$. Electrophoretic mobility shift assays (EMSAs) were performed using wild type Rep68 and the nicking mutant Rep68Y156F as a control. This mutation has been shown previously to have no effect on origin binding or unwinding and is thus consistent with a catalytic endonuclease mutant (19, 22). Both E83C and E164C retain wild type origin-binding affinities in the absence of Mn$^{2+}$, demonstrating the independence of this activity from Mn$^{2+}$ (Fig. 6A). The same held true for helicase activity; both mutants were able to unwind an AAV2 origin DNA substrate efficiently and independently of Mn$^{2+}$, using Mg$^{2+}$ as the metal cofactor (Fig. 6B). In order to demonstrate that the helicase activity was Rep-specific, a helicase-negative variant, Rep68E83C/K340H, was also included. This variant was purified in the same manner as the cysteine mutants and, as expected, did not possess any helicase activity (Fig. 6B). Taken together, these data demonstrate that Glu-83 and Glu-164 do not play roles in origin binding or unwinding and thus represent catalytic residues with respect to endonuclease activity.

**E83C and E164C Do Not Affect the Ability of Rep to Covalently Attach to Origin DNA**—We tested the ability of E83C and E164C to form covalently attached protein-DNA intermediates characteristic of transsterification directed by the active site tyrosine at the TRS (Fig. 7). Covalent attachment assays illustrate that the formation of stable PDCs correlates to the nicking activity of the mutants in the presence of either Mg$^{2+}$ or Mn$^{2+}$. E83C and E164C are able to produce the PDC in amounts relative to their ability to nick the origin substrate in either Mg$^{2+}$ or Mn$^{2+}$. For instance, E83C is only able to form the PDC in the presence of Mn$^{2+}$. As a control, Y156F, the active tyrosine mutant, shows no propensity for forming the PDC, as the linking tyrosine residue is no longer present. These results demonstrate that the catalytic integrity of nicking mediated by E83C and E164C is apparently unaffected by cysteine substitution.

**DISCUSSION**

Although endonuclease activity mediated by an active tyrosine residue is typically metal-independent, we found nicking activity by the Rep amino-terminal domain to require a divalent metal cation (14). This suggested that the active tyrosine residue did not act alone in mediating catalysis, and an unidentified metal-binding site existed within this domain. We set out to identify the residue(s) that conferred this metal dependence in order to better define the active site of the endonuclease domain. The amino acid sequence of the protein did not reveal any homologies with known endonuclease families to aid us in the prediction of potential metal-binding sites. Thus, we turned to a mutational analysis technique geared toward elucidating residues that interact with divalent metal cations.

The cysteine mutagenesis approach is contingent on the ability of the wild type enzyme to be active in vitro using not only magnesium, but also manganese as the divalent metal cofactor. In our hands, the endonuclease domain variant Rep68N2O8A was only appreciably active in the presence of Mn$^{2+}$ ions, and not Mg$^{2+}$ (14). For this reason, the full-length Rep68 protein was used in this study, because it is able to use either Mg$^{2+}$ or Mn$^{2+}$ for nicking activity. In a multifunctional protein such as Rep, it must be taken into consideration that a chemically extreme substitution could affect the integrity of the protein. This concern was especially relevant in this study, because only evolutionarily conserved residues were targeted. All but one of the variants tested were apparently unaffected in their overall structure by the substitution when tested for endonuclease activity on the dsTRS substrate, which requires both the DNA binding and helicase activity to be intact.

Parvovirus Rep proteins have been associated with the family of rolling-circle replication initiator proteins. Characteristic of this class of proteins is the position of the active tyrosine residue as well as a cluster of histidine residues that are similar to the metal-binding motif used by several metallproteinases (37). Mutational analysis has suggested that this putative “HuH” motif (where “u” is any hydrophobic amino acid) is indeed critical for Rep endonuclease activity (32). The definite role of this motif within the parvovirus non-structural proteins has now been elucidated by the recent publications (25) of the AAV type 5 endonuclease domain crystal structure. The structure indicates that the two histidine residues comprising rolling-circle replication motif 2 act together with glutamate 82 (Glu-83 in AAV2) to directly coordinate the divalent metal ion necessary for endonuclease activity. This confirms our prediction that glutamate 83 in AAV2 coordinates the metal ion.

The AAV5 Rep endonuclease structure also simplified the interpretation of mutations whose phenotypes were less clear. For instance, the difficulty in purifying E6C and its subsequent lack of nicking activity suggested that this residue was critical for maintaining the structural and/or functional integrity of the protein with respect to endonuclease activity. Indeed, it is proposed that glutamate 6 in AAV5 Rep is necessary for the proper orientation and reactivity of one of the active site histidine residues. Also noteworthy was the apparently unaffected
phenotype of AAV2 Rep variants D14C, D16C, and D42C, because alanine substitutions at these positions have been shown to completely abrogate nicking activity (32). Examination of the three-dimensional AAV5 Rep structure revealed that these residues are positioned on the outer surface of the endonuclease domain in relatively unstructured regions. Asp-42 has been implicated previously in Rep oligomerization (38), and it remains possible that the endonuclease domain acts as a multimer, as has been suggested (23). This notion is supported by the fact that elimination of a charged residue in this position (i.e., alanine substitution) could result in the loss of oligomerization. Although no evidence has directly implicated Asp-14 and Asp-16 in such a role, these residues may also contribute to protein-protein interactions, based on their positions within the endonuclease structure. Alternatively, alanine substitutions at these positions may disrupt the overall folding of the protein leading to the observed loss in activity, whereas cysteine substitution did not.

Although our data clearly supported a direct role for Glu-83 in metal binding, interpretation of the Glu-164 data was less straightforward. It appeared counterintuitive that cysteine substitution of Glu-164 should alter the divalent metal cation preference of Rep, whereas alanine substitution barely affected nicking at all. Based on these data alone, we could not rule out the possibility that Glu-164 may have a function within the active site. Not surprisingly, the AAV5 crystal structure reveals that Glu-161 (equivalent to Glu-164 in AAV2) is in very close proximity to catalytic residues of the enzyme (<5 Å). It is possible that cysteine in this position slightly disrupts the local environment of the metal-binding site so that it favors interaction with Mn$^{2+}$ over Mg$^{2+}$. We note that in AAV5 Rep, Glu-161, Lys-157, and the active site tyrosine at 153 lie within the same plane. Although a catalytic role for Lys-157 (Lys-160 in AAV2) has not yet been demonstrated, Hickman et al. (25) suggest that this lysine residue may be able to interact with the DNA substrate based on the three-dimensional structure of the active site cleft. Extending this to Glu-164, especially in light of our data as well as Ozawa and co-workers (32) implicating this residue in integration, there may indeed be a catalytic role for Glu-164 in the active site. These residues together with the active site tyrosine may serve to mediate downstream steps of the integration process that are not yet defined and perhaps not addressed by the current standard nicking assay. Development of a biochemically relevant assay for AAV integration as well as the resolution of the endonuclease domain structure complexed with DNA will aid in our understanding of these
residues in the ability of Rep to mediate this complex reaction. It is to be expected that the active sites of two enzymes from the same family would be highly conserved. The catalytically relevant elements of the HuH motif, Glu-6 and Glu-83, and the active site tyrosine are invariably conserved throughout the endonuclease domains of the parvovirus non-structural protein family. It is noteworthy that AAV5 is perhaps the most distantly related of the known AAV serotypes when compared with AAV2 (26). This disparity is reflected in the Rep proteins themselves. Although the Rep open reading frames of AAV serotypes 1, 3, and 4 are over 90% similar to AAV2 Rep, the AAV5 Rep is only 67% homologous to its AAV2 counterpart. Furthermore, the endonuclease domain of AAV5 Rep is only 62% similar to AAV2 Rep at the amino acid level, consistent with the fact that AAV5 Rep cleaves a different TRS sequence that is not recognized by AAV2 Rep (39). This suggests that small variations within the structure of the respective active sites are likely responsible for the differences in target site specificity between the AAV5 Rep and AAV2 Rep endonucleases. The crystal structure of the AAV2 Rep endonuclease domain will prove useful in elucidating the structural determinants of target site specificity within the respective active sites.

As reviewed by Galburt and Stoddard (40), there are several possibilities as to the role of a divalent metal cofactor in the context of endonuclease activity. However, these possibilities are limited by the fact that direct transesterification by Rep must be mediated by the active tyrosine residue at position 156. Given this constraint, a likely role for the divalent metal ion within the active site may be to activate the hydroxyl group of the active tyrosine for nucleophilic attack at the TRS or to stabilize the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonucleases, stabilizing the transition state intermediate of the reaction. Or, as demonstrated previously (41, 42) for other endonucleases, the metal ion may play a role in target site selection, perhaps as demonstrated previously (41, 42) for other endonuclease active site, serves as a first step toward understanding the role of Rep during the integration process and provides a foundation for further exploring the biochemical role of Rep functions in mediating viral latency.

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