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In Vitro Resolution of Adeno-Associated Virus DNA Hairpin Termini by Wild-Type Rep Protein Is Inhibited by a Dominant-Negative Mutant of Rep

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An adeno-associated virus (AAV) genome with a Lys-to-His (K340H) mutation in the consensus nucleotide triphosphate binding site of the *rep* gene has a dominant-negative DNA replication phenotype in vivo. We expressed both wild-type (Rep78) and mutant (Rep78NTP) proteins in two helper-free expression systems consisting of either recombinant baculoviruses in insect cells or the human immunodeficiency virus type 1 long terminal repeat promoter in human 293 cell transient transfections. We analyzed nuclear extracts from both expression systems for the ability to complement uninfected HeLa cell cytoplasmic extracts in an in vitro terminal resolution assay in which a covalently closed AAV terminal hairpin structure is converted to an extended linear duplex. Although both Rep78 and Rep78NTP bound to AAV terminal hairpin DNA in vitro, Rep78 but not Rep78NTP complemented the terminal resolution assay. Furthermore, Rep78NTP was *trans* dominant for AAV terminal resolution in vitro. We propose that the dominant-negative replication phenotype of AAV genomes carrying the K340H mutation is mediated by mutant Rep proteins binding to the terminal repeat hairpin.

Adeno-associated virus type 2 (AAV) is a human parvovirus that usually requires adenovirus or herpesvirus as a helper to replicate efficiently (5). AAV has a single-stranded, linear DNA genome with terminal repeats at either end that function as origins of replication (9, 22). The left half of the AAV genome comprises the *rep* gene open reading frame, which encodes at least four overlapping Rep proteins (Rep78, Rep68, Rep52, and Rep40) that are required for efficient AAV DNA replication and the production of single-stranded progeny genomes for encapsidation (7, 8, 10, 13, 16, 20, 23, 25). Rep78 and Rep68 are translated from unspliced and spliced RNA transcripts, respectively, initiated from the p_5 promoter at map position 5 (13, 20, 25). Rep52 and Rep40 are translated from unspliced and spliced transcripts, respectively, initiating from the p_{19} promoter at map position 19 (13, 20, 25).

AAV has evolved a novel way to solve the problem of replicating its termini. The terminal repeats are self-complementary and fold over into hairpin structures, which provide primers for DNA synthesis (9, 20, 22). It is then necessary to resolve the ends by making a strand-specific, site-specific, endonuclease cut at the terminal resolution site (TRS), followed by unwinding of the hairpin, so that the ends may be replicated and converted to an extended linear duplex (12, 18, 19). This process has been termed terminal resolution (19).

Both Rep68 and Rep78 bind specifically to the AAV terminal hairpin (2, 11, 12, 15) and can mediate nicking at the TRS in vitro (12, 18). It is therefore believed that terminal resolution in vivo requires either Rep68 or Rep78. Also, purified Rep68 has a DNA helicase activity (12). The terminal resolution activity has been demonstrated in vitro with cytoplasmic extracts from HeLa cells infected with AAV and adenovirus or with extracts from uninfected cells complemented by purified Rep68 isolated from cells infected with AAV plus adenovirus (18, 19). The terminal resolution reaction, nicking at the TRS, and the DNA helicase activity, but not the specific binding of Rep to the hairpin, require ATP (12, 18).

A consensus nucleotide triphosphate binding site in the AAV *rep* gene is highly conserved among the analogous genes of many different parvoviruses (3). A mutant AAV genome, NTC23, in which a lysine at this site was changed to a histidine (K340H) could not replicate when transfected into human 293 cells (8). Furthermore, this mutant was *trans* dominant in vivo and completely inhibited the replication of a wild-type AAV genome when the two genomes were cotransfected at a ratio of 1:1 (8).

We developed two helper virus-independent expression systems for the production of AAV Rep proteins. In one system, the *rep* gene was expressed from a recombinant baculovirus in Sf9 insect cells (15). In the second system, the *rep* gene was expressed from the human immunodeficiency virus type 1 (HIV) long terminal repeat promoter in a plasmid that was then transfected into human 293 cells (1). Both expression systems yielded nuclear extracts containing mainly Rep78 (15). Both wild-type Rep78 and Rep78 with the K340H mutation (Rep78NTP) produced in either system bound specifically to AAV DNA hairpin termini (15). We examined the function of nuclear extracts from either expression system in the in vitro terminal resolution assay.

The in vitro terminal resolution assay was performed with the AAV no-end DNA substrate and HeLa cell cytoplasmic extracts as described previously (19, 21). After a 3-h incubation at 37°C, the assay mix was treated with proteinase K, extracted with phenol-chloroform, ethanol precipitated, digested with *Xba*I and *Pst*I, electrophoresed in a 6% polyacrylamide gel, and autoradiographed. The DNA restriction fragments produced in the assay are shown in Fig. 1. The bands containing the resolved terminal fragment X were cut out, submerged in CytoScint ES scintillation fluid (ICN

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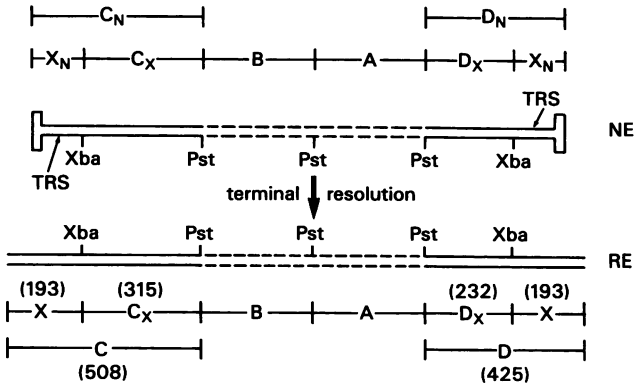

FIG. 1. Structure of AAV NE DNA and products of the terminal resolution reaction. The structure of the AAV no-end DNA substrate is shown before (NE) and after (RE) terminal resolution. The restriction cleavage sites for *Pst*I (Pst) and *Xba*I (Xba) are shown. The sizes of the expected restriction fragments (in base pairs) are indicated by the numbers in parentheses. Fragments containing hairpins have apparent molecular weights that vary with the gel system used (19), but in the gel system used here the apparent sizes of X_N, D_N, and C_N are approximately 130, 365, and 450 bp, respectively. The nomenclature of the diagram follows that of Snyder et al. (19). Fragments A and B (= = =) are not drawn to scale.

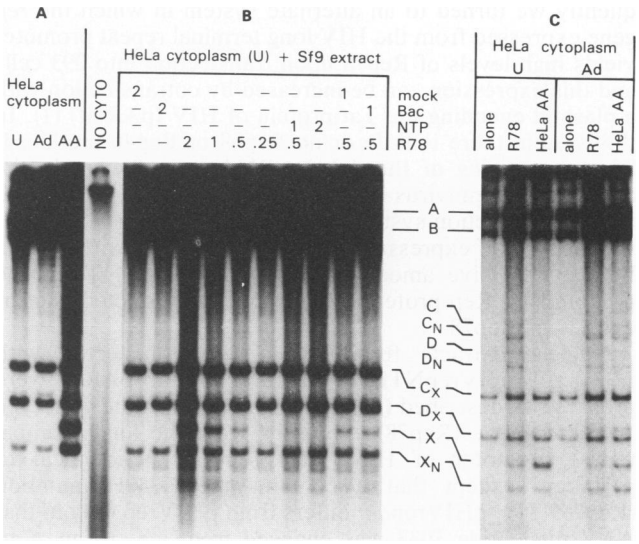

FIG. 2. Terminal resolution assays of Rep78 and Rep78NTP expressed from recombinant baculoviruses in Sf9 insect cells. The positions of restriction fragments described in Fig. 1 are indicated. Fragment X_N represents the unresolved terminal hairpin, and fragment X represents the extended linear duplex termini derived by terminal resolution. (A) Standard terminal resolution assay performed on AAV no-end DNA with cytoplasmic extracts from HeLa cells that were uninfected (U) or infected with adenovirus (Ad) or adenovirus plus AAV (AA). (B) Terminal resolution assays were performed with cytoplasmic extract from uninfected HeLa cells (U from panel A) supplemented with nuclear extract from Sf9 cells (15) that were either mock infected (mock) or infected with wild-type AcMNPV baculovirus (Bac), recombinant baculovirus AcR78 expressing wild-type Rep78 (R78), or recombinant baculovirus Ac78NTP expressing mutant Rep78NTP (NTP). The amounts (in microliters) of Sf9 nuclear extract added to each reaction are indicated at the top of each track. One reaction (no cyto) contained Sf9 nuclear extract (2 μl) from the AcR78 infection but no cytoplasmic extract. (C) Terminal resolution assays were performed with cytoplasmic extract from uninfected HeLa cells (U) or adenovirus-infected cells (Ad) supplemented with no additional nuclear extract (alone), with 2 μl of nuclear extract from Sf9 cells infected with AcR78 (R78), or with 2 μl of cytoplasmic extract from HeLa cells infected with adenovirus plus AAV (HeLa AA).

Biochemicals, Irvine, Calif.), and counted in an LS 3801 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.).

As reported by Snyder et al. (18, 19), the AAV terminal resolution reaction (Fig. 1) could be mediated by cytoplasmic extracts from HeLa cells infected with AAV and adenovirus but not from cells that were uninfected or infected with adenovirus alone (Fig. 2A). In the experiments of Fig. 2, the appearance of fragment X is diagnostic of the conversion of the covalently closed terminal hairpin of no-end DNA to an extended linear duplex structure.

Nuclear extracts containing Rep78 produced in Sf9 cells infected with a recombinant baculovirus complemented uninfected HeLa cytoplasm in the terminal resolution reaction (Fig. 2B). The pattern of radiolabeled bands was identical to that produced with cytoplasmic extracts from HeLa cells infected with both adenovirus and AAV (Fig. 2A). The Rep78-containing Sf9 nuclear extract alone was incapable of terminal resolution in the absence of HeLa cytoplasmic extract (Fig. 2B). Reactions containing nuclear extracts from uninfected Sf9 cells or from Sf9 cells infected with wild-type baculovirus were also unable to complement terminal resolution (Fig. 2B).

The labeling of the resolved terminal fragment (X) is Rep dependent (Fig. 2) and is primarily caused by the synthesis of the complementary strand after the hairpin unwinds (19). The labeling of other fragments is mainly the result of nonspecific nicking and repair processes (19), although some increased labeling of fragments other than fragment X was noted in the presence of Rep protein.

An Sf9 cell nuclear extract containing Rep78NTP could not complement the terminal resolution reaction (Fig. 2B). Furthermore, when 1 μl of the Rep78NTP extract was mixed with 0.5 μl of the Rep78 extract, terminal resolution by Rep78 was inhibited (Fig. 2B). Similar amounts of nuclear extracts from uninfected Sf9 cells or from Sf9 cells infected with wild-type baculovirus showed no such inhibitory effect (Fig. 2B). This suggested that Rep78NTP was *trans* dominant in vitro.

The previous studies of AAV terminal resolution used purified Rep68 or cytoplasmic extracts derived from HeLa cells infected with both adenovirus and AAV (12, 18). It remained possible that the Rep protein had to be modified by the helper adenovirus in order to perform its role in terminal resolution. The experiment in Fig. 2C shows that terminal resolution activity could be complemented by Rep78 equally well with extracts from uninfected or adenovirus-infected HeLa cells. Since the Rep78 was produced in Sf9 cells in the absence of any adenovirus gene products, the terminal resolution activity of Rep78 does not require the helper adenovirus to modify either the Rep protein itself or any of the components of the cytoplasmic or nuclear extracts. This is consistent with evidence that herpesviruses can provide helper functions for AAV DNA replication (5, 17) and that under some conditions helper-free replication of AAV can occur (17, 26).

Further analysis of Rep78 and the apparent *trans* dominance of Rep78NTP with Sf9 nuclear extracts was difficult, because increased amounts of these extracts appeared to have high levels of nonspecific nuclease activity. Conse-

quently we turned to an alternate system in which the *rep* gene expressed from the HIV long terminal repeat promoter yields high levels of Rep78 upon transfection into 293 cells and this expression can be increased by cotransfection with a plasmid encoding the Tat protein of HIV (pARtat) (1). In this system there is little or no Rep68 or Rep40 produced, because splicing of the AAV mRNA is inefficient in the absence of adenovirus infection (1, 24). Rep52 is expressed in this transfection system from the AAV p_{19} promoter (1), but the level of expression is low because of downregulation by the excessive amount of Rep78 (1, 4, 13). Thus, the predominant Rep protein species in the transfected nuclear extracts is Rep78.

Nuclear extracts from 293 cells cotransfected with pHIVrep, pHIVrepNTP, or pHIVrep*am* and pARtat were prepared as described (15). pHIVrep contains the wild-type AAV *rep* gene (Rep78) expressed from the long terminal repeat promoter of HIV. pHIVrepNTP is identical to pHIVrep, except that it contains the K340H mutation (Rep78NTP). pHIVrep*am* differs from pHIVrep only in that AAV nucleotide 1033 was changed from a C to an A to produce an amber termination codon at that site in the *rep* gene (1, 6). pARtat contains the first exon of the sequence encoding the HIV transactivator protein (Tat) expressed from the long terminal repeat promoter (1). pGEM4Z (Promega Corp., Madison, Wis.) was used as control DNA. All plasmids were amplified in *Escherichia coli* DH5 (GIBCO/BRL) and purified twice in cesium chloride gradients.

The amount of Rep protein in nuclear extracts of transfected 293 cells was quantitated first by immunoblotting as described previously (13, 15, 25) with a rabbit antibody (anti-Rep78.93) raised against Rep78 expressed in *E. coli* (25). The secondary antibody was ^{125}I-labeled goat anti-rabbit immunoglobulin G (500 Ci/mmol) (NEN Du Pont). Serially diluted extracts were analyzed, Rep protein bands were cut out, and radioactivity was counted in a Gamma 5500 gamma counter (Beckman Instruments). For this estimation we assumed that the polyclonal anti-Rep78.93 antibody recognized both wild-type and mutant proteins equally (8, 15, 25). Comparing samples within the linear range, by interpolation, we calculated that the Rep78NTP extract contained 8.2 times as much Rep protein per microliter of extract as did the Rep78 extract. The higher level of expression of Rep78NTP in the transfected cell extracts reflects the inhibition, by Rep78 but not by the mutant protein, of the inductive effect of Tat on the HIV promoter (1).

As a second means to assess the relative amounts of wild-type and mutant Rep protein in the transfected 293 cell nuclear extracts, we assayed a function that is not ATP dependent (2, 11, 12, 15). Gel mobility shift assays with ^{32}P-labeled AAV terminal hairpin DNA (11) and serial dilutions of nuclear extract were performed as described before (15). The bands containing bound and unbound radiolabeled hairpin DNA were cut out and counted by using liquid scintillation as described above for the terminal resolution assay. We determined the percentage of ^{32}P-labeled DNA bound in protein-DNA complexes versus the amount of extract to ascertain the linear range of the assay. When the Rep78 and Rep78NTP extracts were mixed at levels within the linear range, the amount of bound hairpin DNA was additive (14). Comparing samples within the linear range, we calculated that the amount of hairpin binding activity in the Rep78NTP extract was 10-fold higher than that in the Rep78 extract. This corresponded well with the difference of 8.2-fold in the amount of protein determined in the immunoblot assay. Figure 3 shows a plot of the relative amount of Rep protein (using the adjustment factor of 8.2 determined from the immunoblots) versus the proportion of hairpin DNA bound in protein-DNA complexes. This indicates that the abilities of Rep78 and Rep78NTP to bind to the hairpin are equivalent. The difference in the relative amounts of Rep78 and Rep78NTP in the extracts (10 versus 8.2) as determined by the two assays might represent a slightly higher binding affinity for Rep78NTP but more likely reflects minor variability within each of the two assays.

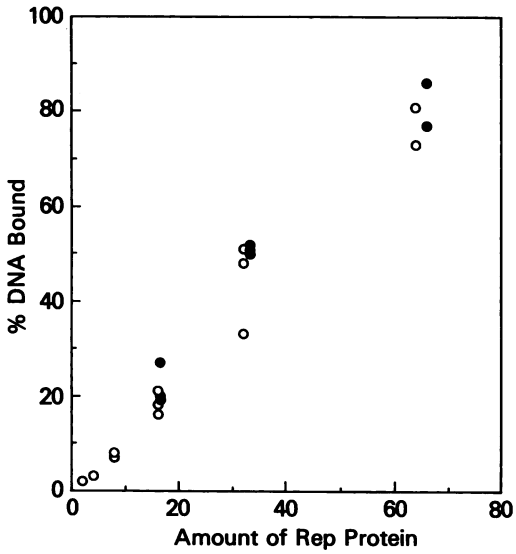

FIG. 3. Comparison of hairpin terminal repeat DNA binding by Rep78 and Rep78NTP. The amount of Rep78 and Rep78NTP protein in nuclear extracts of 293 cells transfected with pARtat plus pHIVrep or pHIVrepNTP was quantitated from immunoblots as described in the text. Various amounts (in arbitrary units) of Rep78 or Rep78NTP extracts were assayed by gel mobility shift for binding to ^{32}P-labeled AAV terminal hairpin DNA, and the percentage of bound DNA was determined as described in the text. ○, Rep78; ●, Rep78NTP.

In the terminal resolution assay, nuclear extracts from 293 cells transfected with pHIVrep complemented uninfected HeLa cell cytoplasm in a manner similar to that of nuclear extracts from 293 cells infected with both adenovirus and AAV (Fig. 4A). The amount of label in the resolved termini (fragment X) was about twofold higher with the transfected cell extracts than with the infected cell extracts, reflecting the higher amount of Rep protein in the transfected cell extracts. Terminal resolution was not detected with nuclear extracts from uninfected or untransfected 293 cells or from cells that were only infected with adenovirus (14). Reactions containing nuclear extracts from 293 cells transfected with pARtat plus either the control plasmid pGEM4Z or pHIVrepNTP were also incapable of terminal resolution (Fig. 4B).

The complementation of terminal resolution by the Rep78 nuclear extract from transfected 293 cells (Fig. 4A) was quantitated by cutting out the part of the gel containing the resolved terminal fragment and counting the amount of ^{32}P. The assay was linear in the range of 62 to 500 nl equivalents of added nuclear extract (Fig. 4A). To analyze the inhibition by the mutant Rep78NTP (Fig. 4B), we complemented the terminal resolution assay with 250 nl equivalents of Rep78 extract, which we designated as a relative amount of 1 with

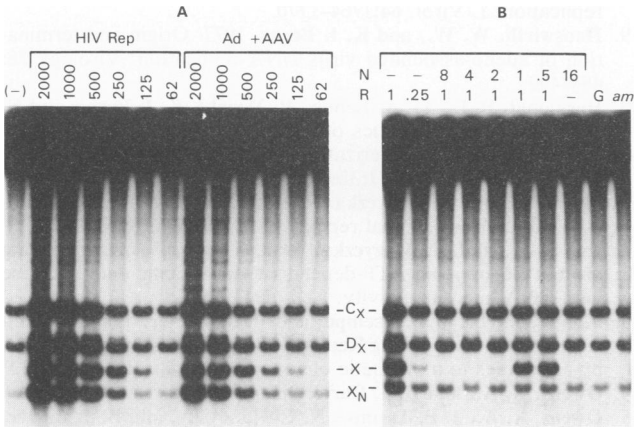

FIG. 4. Titration of the *trans*-dominant effect of Rep78NTP in the terminal resolution assay. Assays contained uninfected HeLa cell cytoplasmic extract and were supplemented with nuclear extracts from 293 cells. (A) Assays contained various amounts (as indicated in nanoliter equivalents at the top) of nuclear extract from 293 cells transfected with pARtat plus pHIVrep (HIV Rep) or infected with adenovirus plus AAV (Ad + AAV) or no nuclear extract (−). (B) Assays were supplemented with various amounts of nuclear extracts from 293 cells transfected with pARtat plus pHIVrep (R), pHIVrepNTP (N), pHIVrep*am* (*am*), or pGEM4Z (G). For R and N the numbers at the top represent the relative amounts of Rep78 and Rep78NTP, respectively, in the extract (determined as described in the text). For R, a relative amount of 1 is equal to 250 nl equivalents of the same extract as shown in panel A. For N, a relative amount of 1 represents the amount of Rep78NTP that gives an equivalent signal in immunoblots and an equivalent amount of band shift activity (Fig. 3) as 1 relative unit of R. For lanes G and *am*, the assays contained 2 μl of nuclear extract. The positions of the terminal restriction fragments are indicated between the panels.

respect to Rep protein. This amount of Rep78 gave less than one-quarter of the maximum activity seen in Fig. 4A and thus represents apparent substrate excess. In the experiment of Fig. 4B, a relative Rep78NTP/Rep78 ratio of 2:1 gave 98% inhibition of terminal resolution and a 1:1 ratio gave 66% inhibition. Even a ratio of 0.5:1 gave 59% inhibition. This experiment indicates that the mutant was inhibitory at relatively low molar ratios of mutant to wild-type protein at apparent substrate excess.

It should be noted that in the experiment of Fig. 2 with baculovirus extracts a twofold excess of Rep78NTP extract over Rep78 showed a less impressive inhibition. However, this is because in the Sf9 extracts (in contrast to the 293 cell extracts) the amount of Rep78NTP protein expression was significantly less than the amount of Rep78 (15) and the amounts of Sf9 extracts were not adjusted for equivalent amounts of Rep protein.

Rep78 and Rep68 differ from Rep52 and Rep40, respectively, by an additional 224 amino acid residues at their amino termini. Previous observations suggested that Rep52 and Rep40 are not required for AAV DNA replication (7) and do not participate in terminal resolution (11). We asked whether the amino-terminal portion of Rep78 and Rep68 was sufficient for any functions. 293 cells cotransfected with pHIVrep*am* plus pARtat contained a truncated Rep protein 237 amino acid residues in length that could be detected in immunoblots (14). The amount of antibody bound to the Rep*am* protein per microliter of extract was about equal to the level bound to Rep78 in the wild-type extracts. Since we know that at least one immunogenic epitope is deleted in this truncated protein (6, 13), the amount of Rep*am* protein may be underestimated in this assay. Nevertheless, extracts containing the Rep*am* protein were incapable of producing specific shifted bands in the AAV hairpin binding assay (15), failed to complement uninfected HeLa cell cytoplasmic extracts in the AAV terminal resolution assay (Fig. 4B), and did not interfere with terminal resolution by Rep78 (14). Although it appears that all or part of this region is necessary for DNA binding and terminal resolution activity, it is clearly not sufficient.

In the experiments reported here we have recapitulated the in vivo *trans* dominance of the K340H mutation in an in vitro terminal resolution reaction. These results indicate that the replication deficiency of this mutant is due to the inability of the mutant Rep78 or Rep68 to perform critical functions in the resolution of AAV terminal hairpins. It has been previously demonstrated that ATP is required for terminal resolution (18, 19). It has also been shown that Rep68 requires ATP either as a substrate, as a cofactor, or as an energy source for both its TRS endonuclease and helicase activities (12). Both of these activities may be involved in AAV terminal resolution (12, 18, 19). Our data suggest that the replication *trans* dominance of the K340H mutant in vivo is due to inhibition of wild-type terminal resolution by mutant Rep78 or Rep68.

The region of the AAV hairpin protected by extracts from cells infected with both adenovirus and AAV is larger than can be accounted for by the binding of a single Rep protein (2, 11). This, combined with the observation that Rep78 and Rep68 produce multiple bands in gel mobility shift assays (2, 11, 15), implies that multiple Rep proteins bind to each hairpin. If a Rep multimer is required for the formation of a stable protein-DNA complex, one method of negative dominance would be the formation of heteromultimers that could not bind DNA. This mechanism for *trans* dominance seems unlikely, because our experiments show that Rep78NTP can bind to the hairpin DNA as well as Rep78 and that mixtures of Rep78 and Rep78NTP show additive binding activity.

These observations would also tend to eliminate the possibility that Rep78NTP excludes Rep78 from the hairpin by virtue of a higher binding affinity. Even if the mutant did have a slightly higher affinity for the AAV hairpin, this alone would not explain our results, because the *trans* dominance experiments were performed with an apparent excess of no-end DNA substrate.

If terminal resolution requires a multimeric complex of Rep78 or Rep68 bound to the terminal hairpin, then the negative dominance could readily be explained by the formation of nonfunctional heteromeric complexes on the hairpin. These heteromeric complexes might either form in solution and then bind to the hairpin or form directly on the hairpin by successive binding of individual Rep molecules. More complex models would require that a multimeric complex of Rep78 or Rep68 forms on the hairpin and that an additional molecule of Rep might then recognize the complex and mediate the site-specific nicking reaction at the terminal resolution site. According to this model, a mutant Rep might recognize and bind to the complex (perhaps more tightly than the wild-type Rep) but not mediate the nick. In any case, the titration experiment of Fig. 4B provides functional evidence that is consistent with models in which several Rep proteins form a complex on the terminal hairpin.

The biochemical studies of Rep78 and Rep68 indicate that each protein can independently mediate terminal resolution in the presence of cellular cytosol. However, since Rep78

and Rep68 differ with respect to the presence (in Rep78) and the absence (in Rep68) of a very hydrophobic carboxyl-terminal domain, they might be expected to have some different properties. Nevertheless, AAV mutations that specifically alter or delete either Rep78 or Rep68 have similar effects on AAV DNA replication in vivo. In either case, the level of AAV DNA replication is decreased about 5- to 20-fold (10, 23, 24). Thus both proteins seem to be required for maximally efficient replication. It is conceivable that each performs different functions at some subsequent step of AAV DNA replication such as melting of the extended duplex to allow the next round of replication or during DNA strand elongation or displacement or perhaps in packaging.

It is difficult to determine whether the extent of *trans* dominance of the K340H mutation in vitro fully accounts for the observed magnitude of inhibition in vivo. In the in vitro assay, a 1:1 ratio of mutant to wild type gave about a 50% inhibition (Fig. 4B). In the in vivo assay a 1:1 input ratio of mutant to wild-type AAV genomes gave complete inhibition of DNA replication (8). This may reflect one of two possibilities. First, the mutant protein may be overproduced in vivo, in which case we cannot distinguish mutant from wild-type protein. Second, the mutant protein may be inhibitory at other stages of the AAV DNA replication cycle that are not reflected in the in vitro assay.

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