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Review

The enigmatic adeno-associated virus: Something old, something new

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ABSTRACT

Since the isolation of adeno-associated virus (AAV) in 1965 as a defective parvovirus found in monkey cells infected with a simian adenovirus, much information has accumulated on the biology of the AAV family of small single-stranded DNA viruses in cell culture. In contrast, very little is known about the natural life cycle in humans and how AAV persists in a high proportion of individuals in a form that has not been associated with overt disease. This review focuses on the findings which suggest that the AAV replication program employs a two-tier strategy. When helper viruses such as adenovirus or herpesvirus are available for coinfection, abundant productive replication ensues. In the absence of such helper viruses, AAV is still capable of replication but at a much lower level, under conditions where viral gene expression is limited by the cellular response to DNA damaging agents and other regulators. It is suggested that it is this basal level of replication, just sufficient for survival in populations of cycling cells, that enables AAV to persist in humans as a non-cytopathic infection. Discussion on the AAV natural life cycle is particularly pertinent since gene delivery vehicles based on AAV have now emerged as clinically-relevant vectors for human gene therapy applications.

KEYWORDS: AAV, DNA damage response, cellular regulators, persistence in humans

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INTRODUCTION AND BACKGROUND

The parvoviruses (*Parvoviridae*) comprise a large family of small single-stranded DNA viruses that have been isolated from many hosts, extending from mammals through birds, reptiles and insects down to crustaceans. The taxonomic division of the parvovirus family into sub-families and genera is based primarily on the host range, phylogenetic lineage and DNA structure [1, 2]. In biological rather than taxonomic terms, the vertebrate parvoviruses are divided into 2 main groups; the autonomously replicating viruses and the helper-dependent adeno-associated viruses (AAV) whose abundant productive replication requires coinfection with helper viruses such as adenovirus or herpesvirus (reviewed in [3]). The distinction between the autonomous group and the helper-dependent AAV is not sharply delineated. The replication autonomy of vertebrate parvoviruses depends on the type of host cells, their physiology and their cell-cycle status at the time of infection [4, 5]. For example, goose parvovirus replicates autonomously in cultures of dividing fibroblasts but replication requires coinfection with a duck herpesvirus when cells are in a stationary phase [6], and other autonomous parvoviruses require a coinfecting helper virus in cells that are otherwise non-permissive [7, 8]. Another aspect of the blurred distinction between the autonomous and helper-dependent parvoviruses is that although high-level productive replication of AAV requires coinfection with adenovirus or herpesvirus (HSV-1 unless otherwise noted) a low level of autonomous, helper-independent replication occurs in some cells under conditions that will be discussed in the first part of this review.

The AAV genome (sero-type 2, unless otherwise noted; Gene Bank accession NC_001401) is a 4679 nucleotide single-stranded DNA with two main open reading frames encoding replication (Rep) and structural (Cap) proteins [9]. Expression of the corresponding *rep* and *cap* genes is controlled by 3 promoter elements, denoted by their genomic positions as p5, p19, and p40. The reading frames, sequence-encoded regulatory motifs and promoter regions are flanked by 145 nucleotide inverted terminal repeats (ITR). One hundred and twenty five nucleotides of the ITR comprise an imperfect palindrome that can form a duplex hairpin structure; the remaining 20 nucleotides, known as the D-sequence, do not participate in the formation of the hairpin structure [9]. The ITRs contain the *cis*-acting replication origin and packaging signals essential for viral replication; the Rep and Cap proteins can be provided *in trans*. This separation between *cis*-acting and *trans*-acting elements of the AAV genome has enabled the development of recombinant AAV (rAAV) vectors such that a foreign DNA flanked solely by the viral ITRs can be replicated and packaged in cell culture under conditions where the Rep and Cap proteins are provided *in trans*, together with adenovirus helper proteins. Such AAV-based recombinant vectors have established themselves as prime vehicles for human gene therapy applications (reviewed in [10-12]). Studies with rAAV vectors have also contributed to our basic understanding of the early events in the wild-type (wt) AAV infection leading to conversion of the incoming single-stranded DNA into a duplex template for viral gene expression. However, it should be noted that the vector genome lacks the *rep* gene whose expression is required for the management of replication-related events, including the specificity of chromosomal integration.

Although a prevalent and persisting human infection, AAV has never been associated with any disease. AAV was first discovered as a small defective virus in lysates of Rhesus monkey kidney cell cultures infected with a simian adenovirus [13], and most investigations on the AAV life cycle continue to be confined to studies in cell culture. The initial events of cell entry, trafficking, uncoating, intra-nuclear conversion to a double-stranded DNA for transcription (second-strand DNA synthesis), viral gene expression, replication and subsequent production of progeny have been reviewed in detail elsewhere [14-16]. In addition to the efficient helper viruses adenovirus and herpesvirus, some other unrelated viruses such as the human papilloma virus and simian virus 40 (SV40) can also provide helper functions, albeit at a lower level (see section 4.5). AAV can persist in cell culture by Rep-dependent chromosomal integration which often occurs at a unique site located on human chromosome 19 (reviewed in [17, 18]). A recent report, however, indicates that Rep-dependent integration events cluster at more than one chromosomal site in diploid human fibroblasts [19]. By infection with adenovirus or herpesvirus, AAV can be efficiently rescued from its chromosomally-integrated state in an established cell line [20] or from a transfected plasmid bearing an intact viral genome [21]. At a lower level, integrated AAV in HeLa cells can be rescued by apoptosis [22].

In this review, some aspects of AAV biology in cell culture that can serve as models to explain lifelong viral persistence in humans will be discussed. The discussion focuses mainly on (a) the evidence for and significance of a low, basal level of autonomous AAV replication, (b) the cells and tissues that are naturally permissive for AAV, (c) the linkage between permissiveness for AAV and the DNA damage response, (d) the regulation of viral gene expression by cellular factors that bind to the ITR D-sequence and (e) possible pathways of long-term AAV persistence in humans. In conclusion, it is suggested that the AAV replication program employs a two-tier strategy. When helper viruses are available for coinfection, abundant productive replication ensues and the cell dies. For long-term survival, it is proposed that AAV replicates at a low level in cycling cells, just sufficient for extra-chromosomal persistence under conditions where viral gene expression is kept in check by the DNA damage response and other cellular regulators.

1. The evidence for a basal level of autonomous AAV replication

The published reports of low-level AAV replication in the absence of efficient helper viruses are listed

in Tables 1 and 2. The lists distinguish infections initiated by AAV virions (Table 1) from those initiated by transfection of plasmid-cloned, double-stranded AAV DNA (Table 2). The justification for this distinction is that DNA transfection bypasses early events required for successful virion infections such as entry of the virus particle, trafficking to the nucleus, uncoating and conversion of the single-stranded parental AAV DNA into a double-stranded DNA competent for transcription. Despite this distinction, the finding that an infectious virus can arise from the transfection of plasmid-cloned AAV DNA in the absence of helper viruses, or helper virus-induced functions, adds substantially to the evidence that autonomous AAV replication can occur in cell culture.

Helper-independent AAV replication and gene expression levels vary depending on the cells and their treatment (henceforth pre-treatment) prior to infection (Table 1, columns 1 and 2). In all cases, these levels are but a small fraction (10% or less) of those obtained when AAV is coinfected with adenovirus or herpesvirus. The proportion of cells supporting helper-independent AAV DNA synthesis varies from 1% to 10% in time-dependent experiments with different genotoxic agents [23-25], the higher values being obtained with cell lines transformed by SV40. With time, the number of AAV DNA-synthesizing cells declines, probably due to the AAV-mediated selective lysis of cells pretreated with genotoxic agents [26].

AAV DNA amplification factors, expressed as the ratio of viral DNA extracted from infected cells pretreated with genotoxic agents to that from infected but untreated cells, range from 32-fold to 750-fold [25]. Recently, AAV DNA amplification in a variety of pretreated cell lines has been reinvestigated using a quantitative polymerase chain reaction (PCR) procedure for measuring the relative number of AAV genomes per cell [27]. The maximum AAV DNA amplification factors were found to be only 10-fold higher than those of the untreated infected controls and only around 0.5% of the levels obtained when AAV DNA synthesis was promoted by herpesvirus coinfection. The reasons for the lower levels of helper-independent AAV DNA replication, compared to those reported in the older publications, are not clear. One suggested possibility is that the older AAV stocks, raised by coinfection with infectious adenovirus, may have been contaminated with helper virus products that co-sedimented with AAV in cesium chloride density gradients [27]. Current methods for producing AAV stocks bypass this potential source of contamination by utilizing plasmid-cloned adenovirus helper products rather than infectious virus [28, 29].

Infectious AAV progeny has been quantitated [23, 24] by a Two-Plate assay in which the yield of virus in the extract of the infected culture (Plate 1) is titrated on HeLa/Ad indicator cells (Plate 2) infected with a sufficient quantity of adenovirus to ensure the presence of the helper virus in each cell. Titration on HeLa/Ad indicator cells provides a highly effective amplification step such that even small amounts of progeny generated in Plate 1 can be detected. AAV progeny yields in OD4 cells, a SV40-transformed line of Chinese hamster embryonic origin [30], were documented at the level of 2 x 10^8 infectious units/10^6 cells when the cells were pretreated with UV-irradiation; a yield 100-fold lower than that obtained when the cells were coinfected with adenovirus. Based on the percentage of cells synthesizing AAV DNA in Plate 1, the yield of progeny virus per UV-irradiated producer cell was estimated as 2000 infectious units [24]. In a notable experiment, Yalkinoglu *et al.* [25] infected SV40-transformed Chinese hamster cells with AAV serotype 5 at a multiplicity of infection (MOI) of 1, serially passaged the infected cells 10 times and then treated the passaged cells with the genotoxic agent 7,12-dimethyl-benzanthracene (DMBA) or infected them with herpesvirus. Yields of 10^3 infectious units/ml of cell extracts were obtained from the DMBA-treated cells compared to 10^5 infectious units/ml from the herpesvirus-infected cells. Importantly, no infectious AAV could be recovered from the passaged cells in the absence of DMBA pre-treatment or herpesvirus infection, despite the amplification step inherent in the HeLa/Ad titration procedure. It is likely that at some time during the passaging of the infected cells, AAV-5 DNA underwent chromosomal integration such that the viral genome could then be rescued by genotoxic treatment or helper virus infection.

Transfection of plasmid-cloned AAV DNAs gives rise to AAV DNA replication in pretreated cells

Table 1. Autonomous AAV replication after virion infection.

Cells[1]	Treatments[2]	Replication events [ref][3]
NB-E*, CO631*	DMBA	Capsid antigens [114, 115]
NB-E*, OD4*, 293, Cos7*, CV1, CHO, 41-DD*	HU, DFMO, 'synchronization'	DNA amplification, progeny virus (OD4*) [23]
CHO, L1210, balb3T3c, XP29mal, HeLa, human fibroblasts, CO631* CO60*	MNNG, N-AAAF,m-AMSA, 4-NQO, UV, heat shock, APH, CH, DMBA	DNA amplification, progeny virus (CO631*) [25]
OD4*	UV (254 nm)	DNA amplification, progeny virus (OD4*) [24]
HeLa	HU, Genistein	DNA amplification [87]
293, 293T*, Cos1*	None, 'SV40 coinfection'	Rep expression, DNA amplification [32]
Human keratinocytes	'Induced differentiation'	DNA amplification, progeny virus [35]
293	None	DNA amplification, progeny virus [37]
Human breast cancer	'Synchronization'	Rep expression, DNA amplification [36]
HeLa, U2OS, MRC5, GM847*	Eto, MMC, Hu, IR	Rep expression, DNA amplification [27]

[1]The asterisk (*) refers to cell lines transformed by SV40 and expressing the SV40 T-antigen (Tag) or to cell lines immortalized by other agents and then transformed to express the SV40 Tag. 293 are human embryonic kidney cells transformed by adenovirus-5 DNA and which express the E1a and E1b gene products; 293T* is an SV40 Tag-expressing derivative of 293. CO60*, CO631*, and OD4* are Tag-expressing clonal isolates of a Chinese hamster embryo mixed cell population transformed by SV40. HeLa are cervical cancer cells carrying human papilloma type 18 genes. Spontaneously transformed cell lines are of human (U2OS, XP29Mal), Chinese hamster (CHO), African Green monkey (CV1) and of murine origin (balb3T3/c). 41-DD* is an SV40 Tag-expressing derivative of CHO. Cos1* and Cos7* are SV40 Tag-expressing derivatives of CV1. NB-E* is an SV40 transformed, Tag-expressing human kidney cell line. L1210 is a human leukemic cell line. MRC5 is of human fetal lung fibroblast origin. GM847* is an SV40-transformed, Tag-expressing, human fibroblast cell line.

[2]Prior to infection. DMBA is 7,12-dimethyl-benzanthracene; HU, hydroxyurea; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; DMFO, alpha-difluoro-methylornithine; UV, ultraviolet irradiation; IR, ionizing irradiation; MMC, mitomycine C; Eto, etoposide; N-AAAF, N-acetoxy-2-acetylaminofluorene; m-AMSA, 4'-(9-acridinylamino)methanesulfon-m-anisidide; 4NQO, 4-Nitro-quinoline-N-oxide; APH, aphidicolin; CH, cycloheximide. 'Synchronization' refers to synchronization of the cell-cycle. 'Induced differentiation' refers to keratinocytes differentiating in a raft culture system (see text). 'Induced apoptosis' is described in the text. 'SV40 coinfection' refers to a double infection of Cos1* with AAV and SV40.

[3]Replication-related events reported: Expression of the regulatory replication (Rep) and capsid proteins. DNA amplification refers to both amplification of total AAV DNA as well as amplification of AAV replication intermediates. Progeny virus refers to the generation of infectious AAV virions (see text). The asterisk (*) above the cell line name in brackets indicates that it is a SV40 Tag-expressing cell line which produced infectious progeny. [ref] reference.

and, in some cell lines, to the production of infectious AAV progeny (Table 2). An advantage of initiating the AAV replication cycle with a transfected plasmid-cloned AAV DNA molecule is that concerns about the purity of the infecting viral stocks are allayed. Using the Two-Plate assay, 4 groups have documented the presence of infectious viral particles in the extracts of the

Table 2. Autonomous AAV replication after transfection of plasmid-cloned AAV DNA.

Plasmids[1]	Cells[2]	Treatments[3]	Replication events [ref][4]
pAV2	OD4*	HU	DNA amplification, progeny virus (OD4*) [23]
pAV1, pAV2	CHO, HeLa, CO60*	MNNG	DNA amplification, progeny virus (CO60*) [25]
pAV2	OD4*	UV, 254nm	DNA amplification, progeny virus (OD4*) [24]
pA2Y1	CHO	MNNG, BrUdr	DNA amplification of whole plasmid [33]
pSub201	293	None	DNA amplification, progeny virus (293) [31]
pAV2	Cos1*, HeLa	None (Cos1*) nocodazole, UV	DNA amplification, progeny virus (Cos1*) [32]

[1]pAV1, pAV2, and pSub201 contain complete AAV genomes. pA2Y1 contains an insert of AAV regulatory DNA, nucleotides 1-1040.
[2]See Table 1, footnote 1
[3]BrUdr is bromodeoxyuridine, nocodazole is a mitotic inhibitor. For other abbreviations see Table 1, footnote 2.
[4]Replication events reported [reference]. DNA amplification refers to excision of AAV DNA from plasmids pAV1, pAV2, pSub201 and appearance of AAV DNA replication intermediates. In the cell lines denoted in brackets, the asterisk (*) indicates SV40 Tantigen-expressing cells that gave rise to infectious AAV progeny. Transfection of pA2Y1 led to amplification of the entire plasmid DNA without excision of the AAV insert (see text).

DNA-transfected cells, as evidenced by their resistance to DNase, their sensitivity to anti-AAV capsid antibodies, their buoyant density in cesium chloride density gradients and their capability to initiate infection in HeLa/Ad indicator cells [23-25, 31, 32]. Although transfection of a double-stranded AAV DNA plasmid bypasses early events of the AAV life cycle, as noted earlier, the demonstration that infectious AAV virions can arise from plasmid-cloned AAV DNA is compelling evidence that at least a part of the AAV life cycle in cell culture can be completed in the absence of a helper virus.

All but one of the transfection experiments listed in Table 2 utilized cloned plasmid DNA that contained a complete copy of the AAV genome. The interesting exception is that a plasmid containing only the non-coding regulatory AAV DNA, comprising the left hand ITR and extending up to the p5 and p19 promoters (nucleotides 1-1045) was found to undergo DNA synthesis in Chinese hamster ovary cells pretreated with a genotoxic agent [33]. Density labeling with bromodeoxyuridine and DpnI resistance of full-length plasmid DNA were consistent with a semi-conservative mode of DNA replication. Restriction enzyme analysis of the products made in cell-free reactions primed with extracts of the pretreated cells indicated that DNA synthesis had initiated preferentially at the AAV DNA insert, although the insert was not excised. Plasmids which lacked the AAV DNA insert did not replicate in the pretreated cells. These results are noteworthy in that they demonstrate that the AAV left hand DNA segment, nucleotides 1-1045, can be activated by the pretreated cell to initiate DNA synthesis in the absence of the viral Rep proteins.

2. Some cells are naturally permissive for AAV in the absence of helper viruses and pre-treatment with genotoxic agents

An important issue in considering the relevance of low-level autonomous AAV replication to the viral life cycle in humans is the question if there are special cells or tissues that are naturally permissive for AAV replication. A particularly relevant report in this respect is the demonstration that AAV replicates autonomously in differentiating

human keratinocyctes [34, 35]. Explanted human keratinocytes are induced to differentiate into squamous epithelial-like cell layers if the submerged cells growing on a rigid collagen matrix are raised to the air-liquid surface. Such 'raft' cultures were originally developed for studying the life cycle of human papilloma viruses [34]. Using this culture system, Meyers *et al*. [35] showed that a full cycle of autonomous AAV replication occurs in human foreskin keratinocytes when the cells, infected as a submerged monolayer, were induced to differentiate into multi-layered 'raft' cultures. AAV replication was demonstrated by the time-dependent appearance of the viral replicative intermediate DNAs in the extracts of the differentiated cell layers and the generation of DNase-resistant AAV progeny virions was demonstrated by the Two-Plate assay described above. Importantly, AAV did not replicate if the infected keratinocytes were maintained as submerged cultures and thus prevented from entering into the differentiation program. The differentiated cells replicating AAV displayed cytopathic-like effects and substantial arrays of 26 nm spherical particles, assumed to be of AAV origin, appeared in many nuclei. There is a need to verify the interpretation of these latter observations with additional electron microscopy under conditions where particles of purified wt AAV are included as size markers and where the formation of specific AAV antibody/ particle complexes is visualized by immune electron microscopy. Using broad-spectrum oligonucleotide primers in PCR reactions, no evidence was obtained for the presence of contaminating helper adenoviruses, herpesviruses or papilloma viruses in the explanted human keratinocytes. On the basis of these observations, it was suggested that AAV may be viewed as "an epithelial-tropic autonomous parvovirus whose life cycle is linked to squamous differentiation" [35].

Recently, Alam *et al*. [36] reported that 3 established cell lines of human breast cancer origin, infected with AAV at the surprising low MOI of 1 infectious unit per 50 cells in sparse culture, gave rise to time-dependent amplification of DNA replication intermediates and the appearance of the viral replication proteins Rep78, 68 and 40. No such permissiveness for AAV was displayed by explanted cells from a normal human mammary gland. The permissiveness of the mammary tumor-derived cell lines correlated with the induction of caspase-dependent and independent apoptosis. A striking feature of these experiments is that the low MOI of 1 unit per 50 cells suggests a 'bystander effect' in which the initially infected fraction of a dividing cell population secretes a permissiveness factor that enhances the spread of the infection.

Several investigators have observed that adenovirus-transformed 293 cells and its SV40-expressing derivative 293T are naturally permissive for low levels of AAV replication [31, 32, 37]. It is not clear if and how the adenovirus proteins E1a and E1b expressed constitutively in 293 cells [38] contribute to cellular permissiveness for AAV infection. African green monkey Cos-1 cells which express the SV40 Tantigen [39] is another established cell line that is permissive for low-level AAV replication [32]. A possible pathway by which the SV40 Tantigen induces permissiveness for AAV is discussed later.

3. Rescue of chromosomally-integrated AAV by apoptosis

After AAV establishes latency in cell culture by chromosomal integration, superinfection of the cells by adenovirus or herpesvirus induces high-level excision and replication, and rare cases of spontaneous rescue have also been noted [20]. Mori *et al*. [22] demonstrated that chromosomally integrated AAV in HeLa cells could also be rescued at a low level by exposing the cells to anti-Fas antibodies, a standard procedure for inducing apoptosis. Viral rescue coincided with the characteristic apoptotic fragmentation pattern of cellular DNA, and rescue and cellular DNA fragmentation were both abolished when the cells were treated with an inhibitor of caspase-8, a trigger of a down-stream cascade in apoptosis. Ten to 20% of the cells exposed to anti-Fas antibody displayed the cellular DNA fragmentation pattern characteristic of apoptosis. Assuming that AAV was rescued only in this subpopulation, the yield was 50-100 infectious AAV particles per apoptotic cell. It is surprising that the role of apoptosis in the rescue of chromosomally-integrated AAV has not been investigated further. Apoptotic rescue of latent AAV in a natural infection might provide an escape mechanism for integrated virus when the cell is faced with

apoptotic death [22]. It might also be a mechanism by which latent AAV could reactivate and continuously infect new cells. A connection between viral latency and apoptosis has been described for the Kaposi-associated herpesvirus (KSHV). In its extra-chromosomal latent form, KSHV replication occurs at a very low basal level in cell culture. However, when the latently infected cells are exposed to a chemical inducer of a caspase-dependent, intrinsic apoptosis pathway, KSHV replication is upgraded and infectious virus is released [40]. An intriguing possibility is that if some cells are latently infected with both AAV and herpesviruses, apoptotic reactivation of latent herpes may also reactivate latent AAV.

4. Permissiveness for AAV infection is linked to the cellular response to DNA damage

The basal level of autonomous AAV replication is a small fraction of that promoted by adenovirus or herpesvirus coinfection. Why then is efficient AAV replication dependent on a helper virus? Or, turning the question around, what curtails AAV replication? Some answers to these questions have emerged from studies indicating that permissiveness for AAV gene expression and replication is linked to the cellular response to DNA damage.

The infectious cycle of many nuclear DNA viruses is influenced, both positively and negatively, by the repair proteins of the DNA damage response (reviewed in [41, 42]). A central player during several stages of the DNA damage response (DDR) is the MRN complex comprising the Mre11, Rad 50 and Nbs1 repair proteins (reviewed in [43-45]). Recent reports (Table 3) have highlighted correlations between MRN deregulation and the enhancement of rAAV transgene expression and AAV DNA amplification. These correlations suggest a common mechanism by which coinfecting viruses, genotoxic agents, mutant cellular DDR signaling and terminal differentiation of the host cell provide helper functions for AAV. The central tenet for the postulated mechanism is that MRN proteins accumulate in AAV nuclear processing compartments, associate with the viral ITR DNA and hinder viral gene expression. Relieving the block of gene expression by displacing the MRN complex from viral DNA, or limiting its association by diminished availability, is a mode of action that is suggested to be the underlying basis of the helper functions [46-48]. The hypothesis is supported by the following.

4.1. The adenovirus E1b55k/E4orf6 helper function involves MRN degradation

In adenovirus infection, the E4 region of the genome functions to degrade the host cell MRN complex via a ubiquitin pathway, a step required to prevent aberrant viral DNA synthesis that generates concatemers [49]. Early reports indicated

Table 3. Impact of MRN deregulation on rAAV vector and wt AAV infections*.

Degradation of MRN proteins, mediated by expression of the adenovirus E1b55k/E4orf6 gene products, correlates with enhanced rAAV transduction, second-strand DNA synthesis and AAV DNA amplification [46, 47].
Down-regulation of MRN proteins, by short interfering RNA targeting, enhances rAAV transduction in cell culture and in animal models [46-48].
Mutant human cells with defective ATM and Nbs1 signaling pathways are more permissive for rAAV transduction [55-57]; ectopic expression of wild-type Mre11 and Nbs1 proteins in the mutant cells diminishes rAAV transduction [46, 47].
Time-dependent down-regulation of MRN proteins in non-dividing, terminally differentiated cells correlates with time-dependent enhancement of rAAV transduction in cell culture and in an animal model [48].
MRN proteins colocalize with nuclear rAAV processing centers and physically associate with the incoming viral ITR DNA [46, 47].
Hydroxyurea treatment of cells, prior to rAAV infection, results in relocation of MRN proteins, diminished binding of MRN proteins to the incoming rAAV ITR DNA and enhanced transduction [47].

*Unless noted otherwise, MRN refers collectively to all 3 of the DNA damage repair proteins Mre11, Rad 50, and Nbs1. Deregulation refers both to down-regulation, via a ubiquitin pathway and to relocation of MRN proteins.

that similar adenovirus gene products might enhance rAAV transduction by promoting the second-strand DNA synthesis that is necessary for converting the incoming single-stranded rAAV genome into a double-stranded DNA template for transcription [50, 51]. The mechanism began to unravel when it was found that the accumulation of double-stranded rAAV vector genomes and transduction correlated with down-regulation of MRN proteins mediated by the adenovirus E1b55k/E4orf6 gene products. Upon rAAV/adenovirus coinfection of cycling cells, MRN proteins relocalize into rAAV nuclear processing centers and physically associate with the incoming rAAV ITR DNA, as indicated by *in vitro* gel shifts [46] and co-immunoprecipitation [47]. Experiments with adenovirus E4 mutants and host cells engineered to express functional or mutant E1b55k products, demonstrated that wt AAV Rep production and DNA amplification are enhanced only in the cells where the MRN complex is degraded. Thus, MRN proteins limit wt AAV DNA synthesis and not just rAAV vector transduction [46].

MRN proteins may hinder rAAV gene expression by more than one mechanism. Lentz and Samulski [52] found that gene expression of self-complementary rAAV vector genomes, which spontaneously fold into double-stranded DNA hairpin structures upon uncoating, was also enhanced by E1b55k/E4orf6-induced MRN degradation. This result is unexpected since self-complementary rAAV vectors bypass the need for second-strand DNA synthesis to form a transcriptionally competent template [53]. The negative impact of the MRN complex in this case may be due to a physical association of Mre11 with rAAV ITR DNA that inhibits gene expression by sequestering the vector DNA to transcriptionally inactive nuclear sites or by forming Mre11-bound ITR DNA complexes that pose a steric block to the binding of transcription factors [52].

4.2. rAAV vector transduction is enhanced in mutant cells with defective MRN signaling pathways and in cells expressing short interfering RNAs targeted against MRN proteins

Patients with the genetic disease ataxia-telangiectasia (AT) lack the ATM protein that manages a major DNA damage signaling pathway [54]. Primary and immortalized cells from AT patients support enhanced rAAV vector transduction in contrast to primary fibroblasts or immortalized cell lines from individuals that do not have AT disease [55-57]. Cells from patients with an AT-like disease in which Mre11 is mutated support enhanced rAAV transduction which can be reversed by ectopic expression of the wild-type protein [46]. Similarly, in cells in which both copies of the Nbs1 protein are mutated, enhanced transduction is diminished when wild-type Nbs1 is expressed ectopically [47]. rAAV vector transduction is enhanced in cells stably expressing a short hairpin RNA directed against Rad 50, compared to cells with unimpeded Rad50 production [46]. Treatment of cycling rat neonatal heart cardiomycytes with siRNA against each of the MRN proteins increases rAAV transduction in an animal model as well as in cell culture [48]. In addition, expression of MicroRNA-24, which down regulates Nbs1, enhances rAAV transduction in Hela cells [48]. Thus, rAAV transduction is enhanced both in cells with mutant MRN signaling pathways and in cells in which MRN components are down-regulated by expression of interfering RNAs.

4.3. rAAV transduction is enhanced in non-dividing terminally differentiated cells

During development, neonatal cells in heart and muscle exit from the cell cycle and differentiate into non-dividing cells (post-mitotic cells). In a series of interesting experiments both in an intact animal model and in cell culture, Lovric *et al*. [48] demonstrated that the natural differentiation process coincides with a time-dependent down-regulation of MRN proteins which in turn correlates with a time-dependent increase in permissiveness for rAAV transduction. It may be recalled that in another setting, wt AAV autonomous replication is induced by a process of differentiation that occurs in skin keratinocytes in raft cultures [35].

4.4. MRN proteins are relocated in cells treated with hydroxyurea (HU)

HU pre-treatment of cells, an inducer of cellular functions for low-level autonomous AAV replication (Tables 1 and 2) and cell-cycle arrest [23] results in a massive relocation of MRN proteins to nuclear centers that are near or overlap with rAAV processing centers [47, 48]. Strikingly, the level

of MRN protein physically associated with rAAV DNA actually diminishes after HU pre-treatment suggesting that HU-mediated MRN relocation displaces the MRN proteins bound to AAV ITR DNA [47]. Other genotoxic agents and inhibitors of DNA synthesis, which enhance rAAV transduction [58, 59] and which induce low-level autonomous AAV replication (Tables 1 and 2) may act similarly by deregulating and relocating the MRN complex.

4.5. In addition to adenoviruses, do other helper viruses deregulate and relocate MRN proteins?

The findings discussed above indicate that MRN ablation, down-regulation or relocation is a feature that is shared by such diverse helper activities as those induced by the adenovirus E1b55k/E4orf6 functions, pre-treatment of host cells with hydroxyurea, cellular functions modified by altered DDR signaling pathways and the cellular differentiation process leading to post-mitotic cells. The question then arises if helper viruses other than adenoviruses also deregulate the MRN complex of repair proteins.

Herpesvirus HSV-1, on an equal footing with adenovirus as an efficient helper virus for AAV, exploits MRN proteins for its optimum productive replication cycle [60, 61]. During coinfection with AAV and HSV-1, the ATM kinase and MRN proteins are phosphorylated and recruited to overlapping HSV-1 and AAV replication centers [62, 63]. As suggested, HSV-1 sequestration of relocated MRN proteins would diminish their availability for association with AAV ITR DNA and thus help to relieve the block of viral gene expression [46, 47].

Human papilloma viruses (HPV) induce helper activities for low-level AAV replication in various settings [32, 64, 65]. Productive replication of HPV, which occurs with the onset of keratinocyte differentiation, requires the MRN component Nbs1 and coincides with the relocation of Nbs1 and Rad 50 to HPV DNA replication compartments [66]. Similar to HSV-1, sequestration of MRN components by HPV would be expected to diminish their access to AAV ITR DNA. However, it has yet to be shown that HPV and AAV DNA processing centers overlap when both viruses replicate in differentiating keratinocyctes.

Cells stably expressing the SV40 Tantigen (Tag) have long been known to be more permissive for AAV replication induced by genotoxic agents compared to Tag-negative cell lines ([23, 25] Tables 1 and 2). Low levels of AAV DNA amplification have been detected in HeLa cells transiently expressing the SV40 Tag [27], and low-level AAV DNA replication has been detected in monkey cells productively infected with SV40 and then super-infected with AAV [32]. There are grounds for proposing that these SV40-mediated helper activities for AAV are linked to an interaction between the Tag and the MRN proteins. Early reports noted that the MRN DNA damage response pathway is perturbed in cells immortalized by the SV40 Tag [67] and that ectopic expression of the SV40 Tag in irradiated cells disturbs the formation of DNA damage response foci containing Mre11 [68]. A physical association between the MRN proteins and the SV40 Tag was first suggested by Wu *et al*. [69] who showed that antibodies to Tag precipitated Nbs1 and Rad50 proteins from extracts of Tag-expressing 293T cells, but not with extracts from the Tag-negative 293 parental cells. Subsequently, it was found that productive SV40 replication in monkey cells activates ATM and ATR signaling pathways and that MRN proteins, together with other DNA damage repair proteins, relocalize with Tag and viral DNA in nuclear replication compartments [70]. During the course of the SV40 lytic infection, the Rad50 and Nbs1 protein levels decline, possibly, it was suggested, due to a proteasome-dependent degradation process that involves the binding of a Tag domain to a member of the ubiquitin CUL7 ligase family [70]. Binding of the Tag to CUL7 has been mapped to Tag residues 69-83 [71]. The proposal of Zhao *et al*. [70] is partly based on the observation that, in contrast to wt SV40, infection of cells with a weakly-binding SV40 dl69-83 mutant did not result in the loss of MRN proteins. However, SV40 dl69-83 alters the growth properties of infected cells [71] such that MRN deregulation could also be an indirect consequence of changes in cell-cycle controls. Not all interactions of the SV40 Tag with CUL7 have been unraveled. Nevertheless, Tag-mediated deregulation and relocation of the MRN complex of repair proteins may be sufficient to diminish MRN availability for deleterious binding to AAV ITR DNA. Since SV40 infections still persist in some human

populations where poor economic conditions and low standards of hygiene prevail [72] it would be important to know if rare spasmodic outbreaks of SV40 infections coincide with outbreaks of localized AAV infection. The possibility of SV40/AAV recombinants being generated under these circumstances would be of concern. In cell culture, the occurrence and structure of such recombinants in monkey BSC-1 cells coinfected with SV40 and AAV have been described [73, 74].

5. AAV infection, in the absence of helper viruses induces an ATR-mediated DNA damage response and cell-cycle arrest

During the investigation of the connection between permissiveness for AAV and the cellular response to DNA damage, it was found that the DDR induced by AAV in the presence of coinfected adenovirus [75] or coinfected herpesvirus HSV-1 [63] differs from that in infections with the helper viruses by themselves. AAV thus seems to be able to modify the interaction between the helper viruses and the host DDR. The question then arises what type of DDR is induced when AAV infects cells in the absence of coinfecting viruses? The findings discussed below indicate that the DDR induced by wt AAV alone differs significantly not only from that induced in coinfections with helper viruses but also from that induced by rAAV vectors in single infections.

The first indication that AAV can perturb the host cell-cycle was the observation that in the absence of genotoxic pre-treatments and coinfected helper viruses, infection of primary cycling cells with AAV led to an arrest in the late S/G2 phases [76]. The severity of this unexpected effect of AAV infection depended on the multiplicity of infection and on the host cells. Pre-senescent human and hamster cells were more sensitive to the AAV-induced growth inhibition compared to immortalized cell lines. No AAV DNA amplification and no cytopathic effects were detected in the growth-arrested cells. UV-inactivated AAV induced growth arrest of pre-senescent cells to the same extent as non-irradiated virus, indicating that the cell-cycle arrest was in response to the infecting virions, or a component thereof, rather than to virus encoded proteins. AAV infection of primary human fibroblasts can also result in a transient block of the G1 phase of the cell cycle. After detachment by trypsin and re-plating, the arrested cells re-enter the cell-cycle and proliferate [77]. Subsequently, Peter Beard and colleagues [78] showed that the cellular response to infection with either untreated or UV-inactivated AAV depend on the p53 status. In p53 null cells, the arrested cells did not recover but died via aberrant mitosis. In cells with an intact p53/p21 pathway, the transient G2 arrest resulted from a DDR signaling pathway in which p53/p21 levels were up-regulated [78]. Up-regulation of p21 in cycling primary cells infected with AAV was also reported by Hermanns *et al*. [79]. In follow-up papers [80, 81], the Beard group showed (a) that it is an ATR signaling pathway that is activated, (b) that ATR-mediated DDR proteins accumulate together with AAV DNA, from both untreated and UV-inactivated virus, in nuclear compartments, (c) that it is only the DNA component of the infecting virions that triggers the response and (d) that in addition to the ITR, a *cis*-acting regulatory segment of the AAV genome is required to trigger the ATR-mediated response. These conclusions stem from the following observations.

Infection of U2OS cells with UV-inactivated or untreated AAV results in phosphorylation of repair proteins RPA32, Chk1 and H2AX and in the accumulation of ATR, RPA and Rad51 in the same nuclear compartments as the viral DNA [80, 81]. Chromatin immunoprecipitation assays indicate that RPA and DNA polymerase delta are physically bound to the viral DNA. The colocalization of RPA and AAV DNA in nuclear compartments, the phosphorylation of Chk1, H2AX and the G2 arrest (abolished when kinase-dead ATR was expressed) are consistent with the activation of an ATR signaling pathway in response to a stalled replication fork [80].

The ATR-dependent DNA damage response and its consequences depend on the type of AAV DNA in the infecting virion [81]. Virions containing the current rAAV vector genomes, which contain only the 145 nucleotide viral ITR flanking the foreign gene of interest, do not induce a detectable ATR response and G2 arrest, even when UV-irradiated. In contrast, when the rAAV vector DNA was extended to include part of the p5 promoter region in addition to the ITR,

AAV nucleotide positions 1-304, an ATR response similar to that triggered by wt AAV infection was documented, as evidenced by H2AX phosphorylation and G2 arrest [81]. The AAV DNA overlapping the p5 promoter region has been shown to contain a *cis*-acting accessary replication element [82-84]. The finding that the AAV ITR DNA needs to be linked to the p5 DNA region in order to induce the ATR replication stress response, suggests that the cell can sense the AAV DNA segment delineated by nucleotides 1-304 as a potential *rep*-independent replication origin. This is in line with the results of Yalkinoglu *et al*. [33] that AAV nucleotides 1-1045, carried in a plasmid, are recognized as an active *rep*-independent replication origin in cells pretreated with a genotoxic agent.

The inactivation of AAV virus by lethal UV-irradiation was originally employed to determine that viral encoded proteins did not contribute to the cell-cycle arrest [76]. Jurvansuu *et al*. [80] showed that UV-irradiation of AAV virions induces covalent intra-strand cross-linking in the viral DNA and that such complex DNA structures persist in the infected cells. The possibility therefore arose that a DDR response might result solely from the presence of the cross-linked aberrant AAV DNA molecules. However, evidence against this possibility derives from the finding that the wt virus induces an ATR-mediated DDR response and G2 arrest similar to that of UV-irradiated virus, albeit at a weaker level [76, 79, 81]. Furthermore, UV-irradiated virions containing standard rAAV genomes fail to induce the response, implying that UV-irradiation by itself is not sufficient [81]. Nevertheless, it is possible that UV-irradiated AAV may induce discrete perturbations of the cellular DDR that were not detectable in infections with untreated virus.

AAV coinfected with helper viruses in cell culture activates different DDR signaling pathways [75, 85, 86]. Some DNA repair proteins, such as those of the MRN complex, pose a barrier to AAV replication. Others, such as the KU proteins of the DNA-dependent protein kinase pathway enhance AAV DNA replication [75, 86]. Thus the question arises if ATR-mediated DNA repair proteins are beneficial or detrimental for AAV. As Jurvansuu *et al*. [80] have documented, UV-irradiated AAV induces a surprisingly wide variety of DNA repair proteins to relocate and accumulate with the viral DNA in nuclear foci. The list so far includes ATR, RPA, Rad51, Rad17, TopPB1, Brca1, BLM, and 9-1-1, but not ATM or Nbs1. It is not clear why the ATM kinase protein and the MRN component Nbs1 were not detected in these experiments. Possibly, the ATR signaling pathway induced by UV-inactivated AAV is so strong that the association of the MRN complex with AAV ITR DNA is masked by the plethora of bound ATR repair proteins [81]. ATR proteins induced by UV-irradiated AAV may pose a barrier to AAV infection or, alternatively, prevent barriers by competing with the deleterious MRN proteins for access to AAV regulatory DNA. A possible experimental approach to these questions would be to determine if and how coinfection with UV-irradiated AAV in excess might modify the course of infection of non-irradiated wt AAV or rAAV vectors.

6. Cellular proteins which bind to the D-sequence in the AAV inverted terminal repeats and their role in AAV gene expression

Arun Srivastava and colleagues [87] discovered a 52-kDa phosphorylated chaperon protein, known as FKBP52, which binds specifically to the 20-nucleotide D-sequence (nucleotides 126 to 145) within the left-end AAV ITR. Only the phosphorylated form binds to the D-sequence. Dephosphorylation, mediated by an inhibitor of tyrosine phosphorylation hinders binding and correlates with helper-independent AAV DNA amplification, and strongly enhances rAAV transduction in HeLa cells. Furthermore, AAV helper activities in HeLa cells (such as adenovirus infection, expression of the adenovirus E4orf6 gene, hydroxyurea treatment and cell-cycle arrest), correlate with dephosphorylation of FKBP52. Collectively, these findings led to the conclusion that phosphorylated FKBP52 bound to the AAV ITR D-sequence blocks second-strand DNA synthesis and viral gene expression [87-89].

FKBP52 is phosphorylated at tyrosine residues by the epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) [90] and dephosphorylated at these residues by a T-cell protein tyrosine phosphatase (TC-PTP) [91]. FKBP52 is also phosphorylated at serine/threonine residues [88] and

dephosphorylated at these residues by protein phosphatase 5 (PP5) [92]. Inhibition of EGFR-PTK signaling not only limits FKBP52 phosphorylation, augmenting transduction from both double-stranded and single-stranded rAAV vectors, but also limits proteasome-mediated AAV virion degradation, via decreased ubiquitination of tryrosine phosphorylated capsids [93]. Hence, EGFRT-PTK signaling and the dephosphorylating enzymes positively influence AAV infection by two pathways; by enhancing the flow of native AAV virions to the nucleus and by hindering the binding of FKBP52 to the left-end ITR of the genome, thus relieving the block of viral gene expression.

FKBP52 is a ubiquitous nuclear protein which in its dephosphorylated form acts as a co-chaperon with heat shock protein 90 in the translocation of steroid hormone receptors and other factors including viral proteins to the nucleus (reviewed in [94]). Apart from its role in regulating the correct transit of AAV virions through the cytoplasm and nuclear pore, FKBP52 may play a broader role in the AAV life cycle by regulating viral gene expression. One possibility to consider is that AAV could exploit the dephosphorylated and phosphorylated states of FKBP52 to switch-on or switch-off viral *rep* gene expression, depending on whether the cellular milieu favors persistence via *rep*-dependent chromosomal integration or persistence as a *rep*-independent episome.

In addition to the D-sequence within the AAV left-end ITR (designated the D[-] sequence) the ITR at the right end contains a 20-nucleotide sequence (nucleotides 4554 to 4535) which is the complement of that within the left ITR and which is designated as the D[+] sequence [9]. By electrophoreses mobility shifts, Qing *et al*. [87] discovered another cellular protein, distinct from FKBP52, which binds preferentially to the D[+] sequence and which was shown to have partial amino acid homology to a NF-kappaB suppressor, a negative regulator of transcription [95]. Chemical modulators of an alternative NF-kappaB pathway enhance rAAV vector transgene expression in HeLa cells [95]. However, the pathways altered by the NF-kappaB suppressor in the context of the wt AAV life cycle have not been investigated.

Very recently, Ling *et al*. [96] reported a series of interesting experiments with modified rAAV vectors where one D-sequence, at either the left or right-end ITR of the vector genome, was replaced with a non-viral DNA that does not bind phosphorylated FKBP52 or the NF-kappaB repressor. Under conditions where Rep, Cap and adenovirus helper proteins are provided *in trans*, the modified rAAV DNA (with one authentic D-sequence, either left or right, retained) replicated almost as efficiently as the parental unmodified genome. This indicates that FKBP52 *or* the NF-kappaB repressor protein is dispensable when rAAV vector replication is supported by adenovirus helper functions. In the absence of adenovirus coinfection, transduction by the mutant rAAV vector was enhanced 6 to 8-fold over that of the unmodified vector with both original D-sequences left intact.

Ling *et al*. [96] have suggested that FKBP52 and the NF-kappaB suppressor might act in concert to minimize viral gene expression during a latent phase of the AAV life cycle. The case for FKBP52 is supported by the data that it can switch-on or switch-off AAV gene expression depending on its state of phosphorylation. The case for the role of the NF-kappaB suppressor is less clear and requires further study. Much of the data on cellular proteins that bind to the D-sequence has been obtained in experiments with rAAV vectors. It remains to be seen how much of that data will be applicable to wt AAV. The experimental approach in which one of the two D-sequences in the rAAV vector genome was substituted with non-viral DNA may not be doable with wt AAV since both authentic D-sequences are required for genome rescue, replication, packaging and integration [97, 89]. Other experimental approaches may need to be explored.

7. AAV persistence in humans

AAV as a natural infection of humans has been investigated by immunological means and by searches for the presence of AAV DNA. Sixty to 80% of healthy adults are seropositive for AAV neutralizing antibodies. Passive maternal transfer at birth is followed by a period of antibody decline during the first year of life, a gradual

increase during childhood, presumably due to infection and then persistence throughout life in a high proportion of adults [98, 99]. The contribution of immunological memory cells to AAV antibody persistence is an open issue. In a screen of peripheral blood mononuclear cells and sera from 55 healthy donors (64.7% positive for neutralizing antibodies to AAV type 1) no correlation was found between AAV-1 capsid-specific T cells and the humoral response; the majority of donors with IgG antibodies to AAV-1 were negative for the T cell response and the majority of donors with capsid-specific circulating T cells (mainly $CD8^+$) were negative for neutralizing antibodies [100]. These studies will need to be extended to include the prevalence and possible contributions of quiescent, virus-specific memory T cell populations that are long-term residents in bone marrow [101].

AAV DNA sequences have been identified in human genitalia [102], and in two samples of testis tissue the viral DNA was reported to be linked to chromosomal DNA [103]. In contrast, the molecular characterization of AAV DNA recovered from human tonsil-adenoid samples indicated a circular double-stranded form, likely persisting as an extrachromosomal episome [104, 105]. In an analysis of biopsy and other samples from 250 individuals, AAV DNA sequences were detected in a wide variety of human tissues, with relatively high levels in liver and bone marrow [106]. Attention is currently focused on readily available peripheral blood as a source for AAV DNA isolation and characterization. AAV DNA has been detected in peripheral blood leukocytes from 2 out of 55 healthy adult donors and in 2 out of 16 haemophilic patients [107]. The prevalence of AAV antibodies was similar (75-83%) in both donor groups. Very recently, a variant AAV DNA sequence was found to occur frequently in the peripheral blood $CD34^+$ stem cells of healthy human adults [108]. Using PCR-based detection for the presence of *rep* gene DNA, 70% of blood samples from 71 healthy donors were found to be positive. The frequency of the variant AAV DNA in the $CD34^+$ stem cell populations, normalized to the single copy ApoB gene, varied from 0.1 to 10 copies per 1000 cells, with the majority of samples analyzed being in the 0.1 to 0.9 copy number range. The AAV genome in the $CD34^+$ stem cells was found to be a natural capsid variant that mapped to a single clade suggestive of a common ancestor. A recombinant AAV vector carrying a reporter gene, packaged in the AAV variant capsid, efficiently transduced human $CD34^+$ stem cells. When transplanted (engrafted) into SCID immune-deficient mice, the transduced human cells continued expressing the rAAV reporter gene throughout the differentiation of the progenitor hematopoietic cells in the bone marrow of the recipient. It will be of considerable interest to determine how AAV with tropism for $CD34^+$ stem cells persists throughout multiple hematopoietic progenitor cell divisions.

There is a precedent for stable chromosomal integration of rAAV vector DNA in transduced hematopoietic stem cells (HSCs). Using a vector containing a gene for the selective recovery of the DNA in bacteria to facilitate sequencing, Han *et al*. [109] documented the long-term survival of the vector DNA in transduced murine HSCs transplanted into irradiated mice. Sequencing of the rAAV vector DNA/mouse genomic DNA junctions revealed insertion sites in several chromosomes. It remains to be seen if the AAV DNA in human $CD34^+$ stem cells, found in a majority of blood donors [108] can be recovered in amounts sufficient for sequence analysis of possible junctions with chromosomal DNA. Still, readily accessible peripheral and cord blood, with their subsets of progenitors and differentiated cells, may well be a promising field for studying AAV latency and persistence in humans.

CONCLUSION

The thrust of this review has been to discuss the reports that in addition to high-level AAV replication, promoted by adenovirus and herpesvirus, a low-level persistent AAV replication can be supported by a variety of factors (Table 4).

There is an emerging concept that the helper-dependency of AAV can be explained on the basis that the MRN complex of DNA damage repair proteins presents a barrier to AAV gene expression and that this barrier can be overcome by adenovirus gene products that downgrade the host cell's MRN complex via a ubiquitin pathway. Correlative studies with rAAV vectors suggest that the MRN-imposed barrier can also be attenuated by

Table 4. Factors leading to persistent low-level AAV replication*.

Chronic infection with inefficient helper viruses such as human papilloma virus or SV40.
Apoptotic rescue of chromosomally integrated virus.
Limited (helper-independent) permissiveness arising from the cellular response to DNA damaging agents, the transformed or oncogenic phenotype of the host cell and terminal differentiation.
Regulation of replication levels by cellular proteins that facultatively bind to the viral ITR D-sequence.

*10% or less of AAV replication levels promoted by the efficient helper viruses, adenovirus and HSV-1.

pre-treatment of the cells with a genotoxic agent, by altered cellular functions associated with mutant DNA damage response pathways and as a consequence of a normal process of cellular differentiation. The connection between deregulation of the MRN complex and a helper virus product has been confirmed genetically only for the adenovirus E1b55k/E4orf6 genes. In the case of the SV40 Tantigen-mediated helper activity, it seems likely that the MRN connection will be supported by further mutagenesis experiments. Tantigen binds MRN components (Nbs1, Rad50) and a member of the ubiquitin CUL7 ligase family active in proteasome-mediated degradation. When the CUL7 binding site on the Tantigen is deleted, the mutant SV40 fails to degrade the MRN complex in monkey cells. It remains to be shown, however, that the binding sites for both CUL7 and the MRN complex occur in the same Tantigen domain and that the mutant Tantigen does not provide helper activities for AAV comparable to those of the wild-type.

It is important to note that cellular permissiveness for AAV and cellular events induced by AAV infection are separate concepts. The linkage between MRN deregulation (to counter the postulated MRN-imposed barrier) and cellular permissiveness for AAV gene expression does not imply that AAV is defective because it cannot overcome a MRN response that it itself might induce. In fact, the evidence to-date is that single infections with wt AAV (or UV-inactivated AAV) trigger an ATR-mediated DNA damage response, reminiscent of that signaled by a stalled replication fork and a connection to MRN deregulation has not been shown. Nevertheless, it would be of interest to see if the AAV-induced ATR repair proteins that bind to the viral DNA hinder the deleterious binding of MRN components, or, conversely, present an additional barrier to infection. This could be investigated by determining if infection with UV-inactivated AAV in excess acts as a helper or as an inhibitor of coinfected non-irradiated virus. Such experiments should be performed both in the presence and absence of helper-virus promoted replication.

The functional division of the vertebrate parvoviruses into separate autonomous and helper-dependent groups needs to be re-considered. Autonomous parvoviruses such as H-1 and MVM exploit coinfection with adenovirus for productive replication in cells that are otherwise non-permissive [7, 8]. Interestingly, the pXX6 helper plasmid for AAV, which expresses the adenovirus E2a, E4orf6 and VA RNA products, strongly enhances replication of a recombinant MVM/H-1 hybrid genome in 293T cells, a finding that has been developed for the production of oncolytic rodent parvoviruses for clinical trials [110]. Another interesting example of an autonomous parvovirus that exploits a helper virus in some circumstances is the human pathogenic B19 virus (B19V). High-level productive infection of B19V is confined to bone marrow erythroid progenitor cells [111]. At a lower level, B19V will replicate in non-permissive 293 cells provided it is coinfected with adenovirus [112]. Furthermore, ectopic expression of the adenovirus E4orf6 gene in 293 cells suffices for B19V DNA replication in the presence of the constitutively expressed E1a and E1b gene products, and specific mutations of the E4orf6 gene strongly suggests that the acquisition of permissiveness for B19V is linked to the degradation of MRN proteins via a ubiquitin pathway [113]. It is intriguing that human B19V and AAV can expand their spectrum of permissive

hosts via a common pathway of MRN degradation. AAV investigators have much to learn from the autonomous parvoviruses.

The relevance of AAV replication, in the absence of adenovirus and herpesvirus coinfection, has been challenged on the basis that the level of replication is too low to be of likely significance in the natural AAV life cycle [27]. However, with so little known about the life cycle under natural conditions, it is difficult to assess what replication threshold is important. AAV persists throughout life in most individuals, as shown by the persistence of neutralizing antibodies and the presence of viral DNA sequences in many adult tissues. In the writer's view, it is unlikely that high-level productive AAV replication, due to occasional external infection with efficient helper viruses, would by itself suffice for life-long persistence. AAV replication promoted by efficient helper viruses results in the death of the coinfected cell. Although the AAV yield is abundant, questions arise as to how the progeny survive and persist when the spread of the lytic helper viruses is curtailed. Terminally differentiated epithelial keratinocytes and skeletal muscle could provide non-dividing host cells for long-term survival of the AAV genome as a double-stranded circular DNA episome. Persistence in cycling cells, in contrast, would necessitate viral gene expression just sufficient to ensure a level of virus production for exit and reinfection. To satisfy these conditions, it is proposed that AAV exploits a two-tier replication strategy. In the presence of efficient helper viruses, AAV replicates to very high levels in cell culture, estimated as 100,000 to 1,000,000 copies per cell, and the cell dies. The alternative AAV strategy (Table 4) is based on replication levels estimated to be 100 to 1000-fold lower and which, in many cases, is helper-independent. Up-regulation of the basal replication level could be kept in check both by barriers imposed by the cellular DNA damage response and by facultative cellular regulators, such as the co-chaperon FKB52, that down-regulates AAV gene expression by phosphorylation-dependent binding to the ITR D-sequence. Chromosomal integration could account for AAV persistence as a DNA genome, with reservoirs of extra-chromosomal AAV virions being supplemented when occasional excision of the integrated genome occurs by natural apoptotic events or occasional superinfection by adenovirus or herpesvirus. In this case too, the proposed two-tier strategy for AAV replication provides a mechanism by which the rescued virus might continue its life cycle by low-level replication in cycling cells.

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CONFLICT OF INTEREST STATEMENT

EW declares no conflict of interest.

ABBREVIATIONS

DDR, DNA damage response; ATM, ataxia telangiectasia mutated kinase; ATR, the ATM-Rad3 related kinase pathway.

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