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Parvovirus Replication

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INTRODUCTION

The parvoviruses are among the smallest and structurally simplest of the DNA animal viruses. The nonenveloped virion has a diameter of 20 to 26 nm and contains a linear, single-stranded DNA genome of approximately 5 kilobases (kb) encapsidated within a simple icosahedral protein coat composed of three proteins with overlapping amino acid sequences. The family *Parvoviridae* (134) has been divided into three genera: parvoviruses, which can grow in dividing mammalian cells of appropriate species origin; adeno-associated viruses (AAV), which usually require a coinfection with either an adenovirus or a herpesvirus (12, 25) for a productive infection in cell culture; and densoviruses, which multiply in insects. Recent studies have suggested that this classification is in need of revision for several reasons. (i) Viruses from all three of the current genera have significant similarities in genome organization; (ii) AAV does not always require a helper coinfection for a productive infection to occur (11, 131, 152, 153); and (iii) the replication of some autonomous parvoviruses has been reported to be greatly enhanced in human cells by adenovirus coinfection (91). Therefore, there is significant overlap at the molecular level in the mechanisms regulating the replication of viruses from all three current genera. On the other hand, detailed new information on genomic locations of transcriptional promoters, splice sites, and signals for polyadenylation, as well as the organization of the terminal genomic sequences, suggests that the parvoviruses might be better grouped into five or six genera or subgenera based on these characteristics. It is the purpose of this article to describe the results of recent studies which have extended our insight into the molecular and cellular mechanisms which regulate the replication of mammalian parvoviruses, in particular, and in some ways relate these findings to the consequences observed upon infection of the intact host.

GENETICS

Genetic analysis of the parvoviruses has been facilitated by the small size of the genome and by the fact that the cloned double-stranded form of the viral genome is infectious when transfected into permissive cells; i.e., upon transfection of a cloned viral genome into a permissive cell, the viral genes are expressed and the viral genome is rescued from the vector, replicated, and then encapsidated to form infectious virus (88, 101, 126, 133). Because the entire sequences of several parvovirus genomes are known (8, 9, 19, 32, 116, 121, 133, 139), it is a straightforward process to create any desired mutant, propagate it in *Escherichia coli*, and then directly assess the phenotype of the mutant in a permissive animal cell line. Thus, it has been possible to do genetic analyses in a fairly straightforward and efficient manner (67, 132, 145).

With the exception of one group of densoviruses (9), all parvovirus genomes have a remarkably similar genetic organization. The left half of the genome encodes regulatory proteins, and the right half encodes structural proteins. Because of the small size of the genome, multiple proteins are encoded in overlapping reading frames. The terminology used to describe the overlapping reading frames differs for the autonomous parvoviruses and AAV. For the latter the regulatory proteins were termed *rep* proteins initially because mutations in the coding region in the left half of the genome blocked DNA replication (67). This is something of a misnomer because we have evidence that these proteins are important regulators of both gene expression and DNA replication. The equivalent proteins of the autonomous parvoviruses are known somewhat more prosaically as nonstructural (NS) proteins. The region in the right half of the genome encoding the coat proteins is called the cap region in AAV and the coat protein region in the autonomous viruses.

All known DNA polymerases require a primer in addition to a template. Because of this requirement, all linear viral DNA genomes have evolved specialized DNA sequences at their termini to maintain the integrity of the 5′ termini. All parvovirus termini so far characterized contain palindromic sequences. For AAV (139) and the human B19 virus (5, 133) the same sequences are present at both termini. This is true as well for the densoviruses (107). However, for the rest of the parvoviruses the 3′ terminal sequence is distinct from

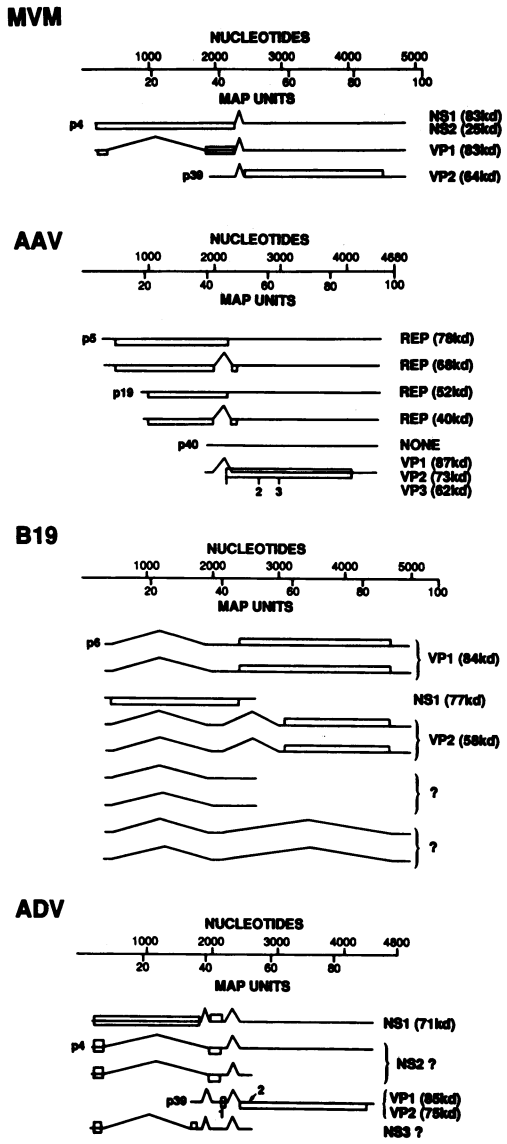

FIG. 1. Genetic and transcriptional maps of four representative parvovirus genomes. The RNAs illustrated are those detected by Northern blots or cDNA cloning. Symbol: □, open reading frames thought to be translated from each RNA. The position of the box relative to the transcript indicates the reading frame used: above, 1; straddling 2; below, 3. Known proteins and molecular masses (in kilodaltons [kd]) are indicated to the right (? indicates that the protein has not been observed). Map positions of promoters are to the left. Introns are indicated by interruptions in the RNAs. See text for details on closely spaced alternative splice donor and acceptor sites of MVM and AAV. Abbreviations: REP, AAV NS proteins; VP, virion structural proteins.

that at the 5′ terminus (Fig. 1). In the case of AAV, the terminal palindrome has 125 bases, but it is only part of an overall inverted terminal repeat of 145 bases (139). As a final oddity, AAV, B19, and densoviruses encapsidate equal numbers of plus and minus strands in separate virions (134). Most autonomous viruses encapsidate primarily the minus strand (complementary to mRNA), but LuIII behaves like AAV in that it encapsidates equal numbers of both strands (48).

All of the parvovirus genomes have a promoter near the left end at map positions 4 to 6 (the map consists of 100 units). With the possible exception of B19 (21, 49, 50, 109), a second promoter at map positions 38 to 40 is found in all parvovirus genomes (58). This promoter initiates the transcript(s) from which the coat proteins are translated. Finally, a third promoter has been identified at map position 19 in the AAV genome (93). Functional polyadenylation signals have been found near the right ends of all parvovirus genomes at map positions 94 to 96 (2, 8, 109, 139). For B19 and Aleutian disease virus, functional polyadenylation signals at map positions 40 to 42 have also been described (2, 109). Appropriate AATAAA signals occur at equivalent sites in other parvovirus genomes, but transcripts corresponding to polyadenylation at these sites have not been found. Diagrams of the genetic maps of the different parvoviruses, illustrating the terminal palindromes and different promoters and polyadenylation signals, are given in Fig. 1. (One group of putative densoviruses in which the regulatory proteins and structural proteins are encoded on opposite DNA strands [9] has been omitted from this discussion and from Fig. 1.)

Gene Expression

The small size of the parvovirus genome has led to the evolution of a variety of strategies for the expression of all of the proteins necessary for a productive infection. The mechanisms involved include multiple promoters, splicing, alternative splice donor and acceptor sites, use of an unusual initiation codon, and proteolytic cleavage.

Regulation of transcription. All parvovirus genomes encode regulatory proteins which affect viral gene expression, including that at the level of transcription. The most extensive data are for transactivation of the p38 promoters of minute virus of mice (MVM) and H-1 and both the p5 and p40 promoters of AAV. Evidence has been found for a temporal regulation of transcription in MVM (35). This is in accordance with the observation that NS protein is required to transactivate transcription from the p38 promoter (51, 119). Rhode has identified a *cis*-active sequence in H-1 DNA that is needed for transactivation (123). The sequence is located between bases −137 and −116 upstream from the TATA box. MVM and B19 p4 promoters are up regulated by their NS-1 polypeptide (50), but there is also evidence of regulation by a structural protein(s). The prototype strain of MVM (MVMp) productively infects mouse fibroblasts in cell culture, but does not replicate in murine T lymphocytes. However, a second virus strain, MVMi, has the reciprocal phenotype, positive for T cells and negative for fibroblasts (96, 137). The molecular basis for the difference in tissue specificity between the two strains has been demonstrated in two ways. By genetic analysis of chimeric MVMi-MVMp genomes, the critical area for productive infection in either fibroblasts or T cells was mapped to the same 237-nucleotide sequence in the middle of the structural protein gene. An additional sequence necessary for productive infection in fibroblasts was mapped near the p38 promoter (55). The second piece of evidence came from experiments in which the results from transfection with the cloned MVMi and MVMp genomes were compared with the results from virus infection (3, 54). Although with virion infection there was a 100-fold difference in the levels of transcript accumulation between productive and abortive infection, with transfection neither the MVMi nor the MVMp DNA clones differed in their ability to yield significant transcript accumulation. In both cell lines transcript accumulation was very low and indistinguishable. The conclusion drawn from these two sets of experiments was that virus transcription (or transcript

accumulation) was particle mediated (54); i.e., it appeared that a structural protein mediated viral regulatory gene expression.

The tissue specificity points to the critical interdependence of viral and cellular regulatory functions, a theme which is constantly replayed throughout parvovirus replication. A transcriptional analysis by deletion mapping of the MVM p4 promoter has identified sequences required in *cis* between nucleotides −55 and −25 relative to the site for initiation of transcription. The critical sequences include the TATA box and a GC box (1). Two proteins from infected-cell lysates, similar to the transcription factor Sp-1, were found to bind to the GC box. Additional sequences that stimulated transcription, but were not essential, were mapped from nucleotides −139 to −55. Similar data have been reported for the B19 parvovirus (20).

Data have also been reported that indicate attenuation of transcription from the p4 promoter (16). In vitro assays have demonstrated a sequence 142 to 147 nucleotides downstream from the MVM p4 promoter that can terminate transcription. The 3′ end of the attenuated transcript is a palindrome followed by a polyuridylate stretch, similar to other reported attenuators. Short RNA species corresponding to the in vitro-attenuated transcript have also been found in both nuclear and cytoplasmic fractions of LA9 cells late after MVM infection.

The regulation of AAV transcription is equally complex. A major difference is that healthy cells are generally nonpermissive for AAV transcript accumulation until infected by a helper adenovirus or herpesvirus or exposed to a toxic chemical or physical agent. Under nonpermissive conditions it is difficult to demonstrate AAV gene expression either by transcript accumulation or by chemical or immunologic detection of AAV proteins. However, it is readily possible to demonstrate at least nonstructural *rep* gene expression under so-called nonpermissive conditions in a variety of biological assays to be described below. In one set of such experiments, a construct was made in which the bacterial *cat* gene was placed downstream of either the AAV p5 or p19 promoter (13). After transfection into HeLa cells both the p5 and p19 promoters could drive the constitutive expression of *cat* in the absence of helper virus. However, cotransfection with a construct containing an intact *rep* gene inhibited *cat* expression, and the inhibitory effect of *rep* could be reversed by adenovirus infection. Therefore, *cat* gene expression was inhibited with only *rep* present, but not with the addition of adenovirus. At the level assayed, the results mimicked the biological phenomena observed with AAV; whether they are exactly comparable remains to be demonstrated. In a similar study Chang et al. (30) mapped two *cis*-active sequences upstream of the p5 promoter which respond to the adenovirus helper function. One corresponds to a sequence found in the adenovirus major late promoter that is activated by E1a expression. The second corresponds to a tandemly repeated decamer (TTTTGCGACA). Removal of these sequences did not affect the ability of the *rep* gene product to inhibit expression from the p5 promoter. Hence we conclude that the mechanism of *rep* inhibition is distinct from the site of action required for the adenovirus helper effect (A. Beaton and K. I. Berns, unpublished data). One possibility is that the function of the TATA sequence itself is inhibited by *rep*, if, indeed, the inhibition is at the level of initiation. However, it is possible that the inhibition observed in these experiments is at a postinitiation level.

Under permissive conditions *rep* has been shown to be a transactivator (84, 147). In Northern (RNA) blots significantly more p40 transcripts than p5 or p19 transcripts are detected. However, nuclear runon experiments have shown that the levels of initiation in a productive infection from the p5 and p19 promoters were equal to that from the p40 promoter. When AAV sequences between map positions 10 and 37 were deleted, transcripts from the p5 promoter accumulated at a level at least equivalent to those observed from the p40 promoter if an intact *rep* gene was present in *trans*. Therefore, there is a *cis*-active negative regulatory sequence which acts at a postinitiation level to suppress the accumulation of the AAV p5 transcript under permissive conditions (84). Presumably this is a mechanism by which the amounts of AAV regulatory protein synthesized are kept to a lower level than those of the structural proteins translated from the p40 transcript. Whether the mechanism by which the *cis*-active negative regulatory sequence works is equivalent to attenuation or alternatively serves to destabilize the p5 (and p19?) transcripts is not yet known.

In summary, parvoviruses stringently regulate their transcription via viral gene products. However, viral transcription is also highly dependent on the intracellular milieu, as indicated above. Cellular effects on viral transcription and gene expression are considered in the next section.

Cellular regulation of gene expression. The autonomous parvoviruses appear to be highly host and tissue specific. For viral replication to occur, the cell must undergo the S phase of growth (118, 135), but clearly gene expression can occur prior to the S phase-dependent phenomenon of viral DNA replication (35). Studies which document the significance of the extent of early gene expression in relation to the cellular phenotype are described in detail below in Suppression of Oncogenicity. A second critical factor appears to be the state of cellular differentiation. Murine teratocarcinoma cells have been reported to be refractory to MVM infection, yet cells from 16-day mouse embryos are permissive, as are several differentiated mouse cell lines (102). These and similar studies have primarily measured viral replication rather than transcription, but it seems likely that regulation of gene expression is a significant factor. Measurements of transcription were described above with respect to MVMp and MVMi (3, 54, 55).

A significant amount of information is available concerning AAV gene expression in different cells. Clearly there is expression of at least the AAV regulatory *rep* gene even under nonpermissive conditions, as outlined in the previous section. However, the effects of *rep* gene expression under nonpermissive conditions appear to be primarily inhibitory for AAV expression and for transcription from promoters of other viral and cellular origins when such promoters are episomal. In different cells the AAV *rep* proteins can have different effects on expression from the p40 promoter, either positive or negative (147). Effects on cellular chromosomal promoters have not been documented. The salient feature of AAV biology is the nonpermissiveness of cells in culture unless exposed to what may be considered toxic conditions. Exposure of several kinds of cell lines to heat, UV irradiation, cycloheximide, hydroxyurea, and chemical carcinogens either renders the cells permissive for viral production or, at least, permits viral transcript accumulation and DNA replication (152, 153). Of course the major toxic exposure in terms of rendering the cells permissive for productive AAV infections is infection of cells with a helper adenovirus or herpesvirus (12, 25). The adenovirus helper functions were the first to be defined in detail. A large number of early adenovirus genes have been implicated, including E1a, E1b, E2a, E4, and the adenovirus VA RNAs (71, 72, 87, 97, 124,

125). The feature in common for all of these adenovirus genes is their effects on viral gene expression. In a coinfection, E1a transactivates AAV transcription: E1b and E4 products work in a cooperative fashion to allow the accumulation of AAV mRNA within a normal time frame (128). E2a is thought to be involved in transport of mRNA to the cytoplasm (71, 97), and the VA RNAs are thought to work at the level of translation (71).

Heat shock of cells prior to AAV infection allows detectable AAV mRNA accumulation (M. Labow, unpublished data). Comparable to that seen with E1a, this type of result suggests that E1a turns on a special class of genes with a common regulatory mechanism and that genes of this class are found in AAV, adenovirus (and other DNA viruses), and human cells. The result, however, does cast some doubt on the notion of a special relationship between adenovirus and AAV; rather, it suggests that the ability of AAV to replicate is a function of the intracellular milieu. If the milieu is such that the right class of genes will be expressed, AAV replication ensues. Therefore, helper virus (adenovirus or herpesvirus) coinfection might be viewed with respect to the effects on intracellular milieu as opposed to the effects directly on the AAV genome; there might then be some conditions under which some cells in culture are fully permissive for AAV replication in the absence of helper-virus coinfection. Indeed, this has been shown to be so. Pretreatment of cells with a variety of agents including hydroxyurea, cycloheximide, UV irradiation, and chemical carcinogens will render at least one line of simian virus 40 (SV40)-transformed hamster embryo cells and one line of human origin (NBE) permissive for productive AAV infections in the absence of a helper-virus coinfection (11, 130, 152, 153). (As an aside, the autonomous H-1 parvovirus does not replicate in human cells unless there is an adenovirus coinfection [91]. Therefore, a full switch of generic phenotype can now be achieved by the appropriate manipulation of the cell culture conditions.) Some of the agents which render cells permissive for AAV replication block the cell cycle at the G_1-S boundary so that an accumulation of gene products necessary for DNA replication may help to achieve permissiveness. Alternatively, or additionally, some of the successful pretreatments involve DNA-damaging agents which induce repair pathways, and it may be that the repair pathways are comparable to those used for AAV replication.

The ability of AAV to replicate in cell culture in the absence of a helper-virus coinfection casts serious doubt on the classification of the virus as either defective or any more dependent than any other virus. By definition, viruses are obligate intracellular parasites, and for all viruses there are both permissive and nonpermissive cells. Our current view is that AAV has evolved a strategy which allows it to establish a stable relationship within the infected cell as long as the cell is healthy. However, if the cell is damaged, productive AAV replication is activated and the virus seeks a new host. This mode of replication would imply a highly evolved form of parasitism. In a more general sense, the concept that the viral infectious process involves both the virus and the host equally is reinforced. The effects of parvoviruses on transformed cells and their ability to establish latent infections will be considered below.

Proteins

The parvovirus genome is among the smallest of the DNA viruses. As demonstrated for other viruses with small genomes (e.g., papovaviruses and hepadnaviruses), the need to encode enough proteins to allow replication requires strategies to allow a given DNA sequence to encode more than one protein. At the first level of organization the parvovirus genome is a model of simplicity. All proteins are encoded in one strand: the left half of the genome encodes regulatory proteins, and the right half encodes structural proteins. Ultimately, the organization is quite complex, because there are two to four overlapping NS proteins and two or three overlapping structural proteins in each case. The strategies used to produce these proteins and their properties are described below.

Regulatory proteins. The parvovirus genome encodes multiple regulatory proteins with partially overlapping amino acid sequences. A common approach is the use of alternative splicing to achieve this. Parvoviruses have the unusual property that both the unspliced and spliced transcripts from a single promoter can serve as mRNAs. The situation is more complex for AAV because the *rep* gene transcripts are initiated from two promoters and both transcripts may either be spliced or remain intact (59, 60, 73). The situation is made even more complex by the possibility that closely spaced alternative splice donor and/or acceptor sites may be used (14, 104). To date, one or two NS proteins of several of the autonomous parvoviruses have been identified by immunological assays (42, 90, 112) and four AAV NS proteins have been identified similarly (100, 115). Additional small open reading frames exist, but proteins corresponding to these have not been reproducibly demonstrated in extracts from infected cells. Nevertheless, the possibility of more NS proteins remains. Alternative splicing in a parvoviral nonstructural gene links a common amino-terminal sequence to downstream domains which confer radically different localization and turnover characteristics (S. F. Cotmore and P. Tattersall, personal communication). The only evidence for posttranslational modification of any of the NS proteins has been the report that the NS protein of several of the autonomous parvoviruses appears to be phosphorylated (43, 103, 113). This modification has not been reported for AAV rep proteins.

As suggested above, the NS proteins appear to play roles in both gene expression and DNA replication. For all parvoviruses the NS proteins transactivate gene expression of the virus. For the autonomous parvoviruses this has been shown at the level of transcript accumulation (MVM, B19, H1) for the p4 and p38 promoters (50, 119) and at the level of initiation of transcription for the p5 and p40 AAV promoters (84). Under nonpermissive conditions, the *rep* protein(s) inhibits gene expression from AAV promoters (13). Under all conditions tested to date, the NS proteins appear to inhibit expression from nonviral promoters (83, 99). It is important to note that the sensitivity of the heterologous promoters has always been tested with promoters that are on plasmids and not in the context of a cellular chromosome. However, there is some evidence that B19 and MVM NS protein may be lethal in some cells (24, 110). AAV has also been reported to perturb the cell cycle in some cells (151). Under conditions of AAV and adenovirus coinfections, adenovirus E1a turns on high levels of AAV NS protein gene expression. This expression in turn inhibits the expression of the adenovirus E1a gene and, hence, subsequent adenovirus early-gene expression; i.e., there may be simultaneous activation and inhibition of different promoters. It also appears that the AAV *rep* protein may negatively regulate gene expression at a posttranscriptional level, most probably at the level of translation (Labow, unpublished).

Most of the data currently available concerning the ability

of the NS proteins to affect gene expression would suggest that most, if not all, of the observed effects are not the consequence of direct binding of the protein to the viral genome. It seems more likely that the NS proteins complex with cellular proteins (e.g., transcription factors) and that the complex then either binds or is prevented from binding. However, this is a poorly defined area in parvovirus replication; little has been reported in detail. There are two instances in which binding of NS protein to viral DNA can be demonstrated. Replicative DNA forms of rodent autonomous parvoviruses with the larger NS-1 covalently attached to the 5′ termini of the duplex structure have been extracted from infected cells and are considered normal intermediates in DNA replication (see below) (44, 62, 117). It has not been possible to detect similar protein-DNA complexes in AAV-infected cells. However, the larger AAV NS protein has been shown in vitro to bind to the AAV terminal repeat if and only if the terminal repeat is folded on itself in the presumed T-shaped hairpin structure (4, 70). Footprinting studies from two laboratories have indicated that the binding is a consequence of conformation rather than primary sequence. Genetic studies have also supported this conclusion (23, 92). Because the terminal repeats of AAV have been implicated in a variety of activities, including both negative and positive regulation of DNA replication (84, 145) and as enhancers of transcription (13), the exact significance of the binding to the terminal repeat is not simple to sort out. Recent evidence has demonstrated that the 68-kilodalton AAV *rep* protein can function in vitro as a site-specific nuclease of the hairpin structure (70a, 136). It can then covalently bind to the 5′ terminus produced (as seen in vivo with the autonomous viruses) and can function as a DNA helicase (70a).

All the genetic studies have demonstrated that the larger NS protein gene is essential for viral replication. Because of the overlapping nature of the regulatory proteins, it is difficult to specifically mutate the smaller species. As shown by the following examples, it is relatively easy to inactivate the larger protein selectively, but this almost inevitably inhibits either replication itself or the specific effect being studied. (i) The AAV *rep* gene inhibits in *trans* the ability of a plasmid carrying the neomycin resistance gene under control of the SV40 early promoter to transform murine cells to resistance to geneticin (G418). A frameshift mutation that specifically inactivates the proteins translated from p5 transcripts (the larger *rep* proteins) does not completely block the inhibitory effect on transformation to G418 resistance in murine melanoma cells, but abolishes the inhibitory effect on transformation in Ltk^- cells (83). (ii) Conversely, mutation of the initiator codon of the p19 *rep* protein(s) to a codon for glycine does not block DNA replication, but does inhibit sequestration of single-stranded DNA (31). However, this phenotype is indistinguishable from that seen with a mutant in the coat protein gene. Therefore, by either positive (leaving only the smaller protein to function) or negative (selectively inactivating the smaller NS protein) approaches it has been difficult to unambiguously define specific functions for the smaller NS proteins in cell culture. Clearly, more sophisticated approaches will be needed to answer this question satisfactorily. However, the demonstrated multiplicity of functions of the *rep* protein is a property that this protein has in common with the regulatory proteins of other small DNA viruses, such as the SV40 T antigen. This multiplicity of functions again seems to have evolved as a way to overcome the constraints of the small size of the genome.

Structural proteins. Parvovirus particles contain two or three different structural proteins of various molecular weights, but with overlapping amino acid sequences. The two larger structural proteins seem to result in most instances from the use of alternative splice sites in the processing of the primary transcripts (MVM uses alternative donors; AAV uses alternative acceptors) (14, 27, 149). However, the smallest virion protein is produced quite differently by the autonomous rodent parvoviruses than it is by AAV. In the former case the middle-sized coat protein, VP-2, is proteolytically cleaved to produce VP-3 (74). In cell culture the ratio of VP-2 to VP-3 varies with the time after infection. AAV has evolved a different approach. VP-2 is initiated at an ACG codon on the same transcript used to produce the smaller VP-3, which starts at a downstream AUG (15). AAV VP-3 represents 80% of the total virion protein mass.

The role of two or three coat proteins with overlapping amino acid sequences is not well understood. The extended NH_2 termini on the larger species may interact with the viral genome. In this context the apparent role of the coat protein(s) in turning on high levels of transcription in a tissue-specific manner in MVM is of interest (3, 54).

Leader sequences in the coat protein RNAs appear to have a regulatory effect on translation. B19 capsid protein production has been reported to be regulated at the level of translation by multiple upstream AUG triplets (111). A leader sequence in the AAV p40 transcript appears to prevent *rep* gene product inhibition of translation (Labow, unpublished).

DNA Replication

As a linear, single-stranded DNA, the parvovirus genome represents a relatively unusual structure in terms of DNA replication. At the most basic level there are two outstanding features. The first is the existence of palindromic terminal sequences which can apparently function as primers for DNA replication and which serve to maintain the integrity of the terminal sequences. The second is that replication proceeds by a single-strand displacement mechanism, so there is no lagging-strand synthesis (64, 79, 129, 132, 140, 141). The current models for AAV and MVM DNA synthesis are shown in Fig. 2 and 3, respectively. A major difference results from the fact that the AAV genome has an inverted terminal repeat, whereas MVM has different palindromic terminal sequences. The latter situation appears to significantly complicate the replication process.

A major question with the AAV scheme is whether the original incoming parental strand can simply fold over the 3′ end to serve as a primer to directly initiate the replication process or whether the inverted terminal repeats first must base pair. The latter possibility is attractive because such a duplex terminus is structurally equivalent to the duplex replicative intermediate from which subsequent rounds of replication are hypothesized to initiate. Recent work has indicated that if the terminal repeat sequences must base pair the ends together, such base pairing need not be perfect (22, 23). This consideration arose because the palindromic sequences in the AAV terminal repeats are inverted during DNA replication, so that the inverted terminal repeats on a strand will not be perfectly complementary if both are not in the same orientation (flip-flip or flop-flop) (139). The inversion of the terminal palindrome occurs with equal frequency at both ends of the genome. This represents another difference with respect to MVM DNA replication. During MVM

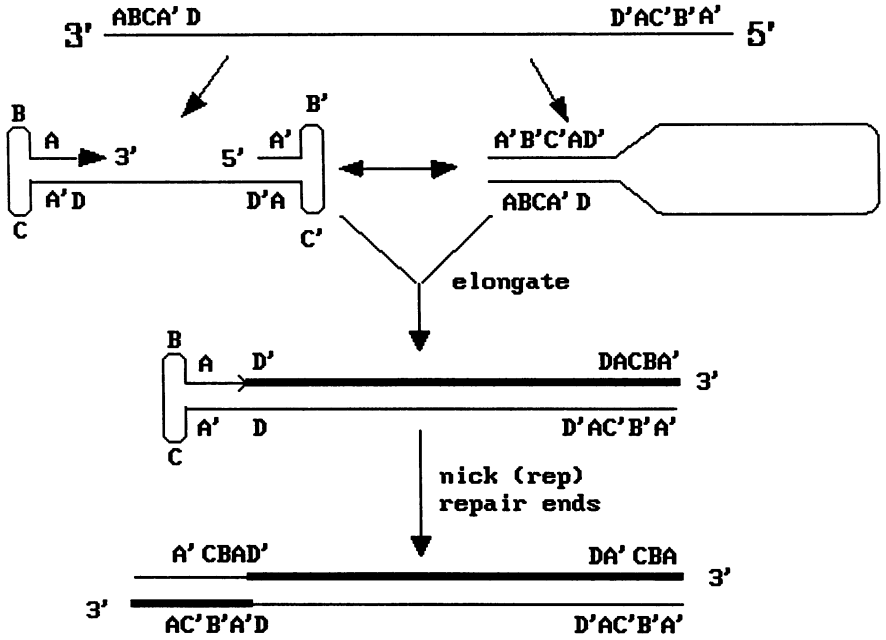

FIG. 2. Model for AAV DNA replication. See text for details. ABCA'D and D'AC'B'A' are inverted palindromic terminal repeats.

DNA synthesis only the 5'-terminal palindrome of the viral strand is inverted (6). The 3' end of the viral strand remains in a single orientation. The original rolling-hairpin model (141) was modified to account for this feature of MVM replication (6).

In the AAV terminal hairpin structure it is possible for almost every base to pair except for the six required to go around the bends in the hairpin and the single base between the two internal palindromes. The ability to form the AAV hairpin structure appears to be more important than the actual sequence (23, 92). However, in the stems of both the 5'- and 3'-terminal palindromes of MVM, there are mismatched regions which are thought to serve as signals for the site-specific nickases and ligases required by the models of replication (7, 41). A significant feature of the MVM model is the requirement for at least a dimeric replicative intermediate. The AAV model neither requires nor prohibits dimeric or larger intermediates. In fact, the longer intermediates have been isolated from AAV-infected cell extracts (140). An additional special feature of the MVM model involves the terminal 24 bases at the 5' terminus. NS-1 is covalently linked to the 5' terminus of the MVM replicative intermediate. When the viral strand is encapsidated, the terminal 24 bases attached to NS-1 extend outside the capsid and can be subsequently cleaved off (45). Presumably NS-1 is functioning as a nickase and possibly has additional functions during replication. This notion is supported by recent studies with the AAV *rep-68* protein (70a, 136). Using in vitro assays involving the putative hairpinned replicative intermediate, investigators have found that a partially purified preparation of the largest AAV *rep* protein (68 kilodaltons) can function as a nickase at the site proposed in the model (on the parental strand at a point opposite the original 3' terminus, i.e., at base 125). In vitro the *rep* protein forms a covalent linkage with the 5' base at the nick site and also functions as a helicase. Other than the NS proteins described above, the DNA polymerase(s) used and ancillary proteins are of cellular origin. DNA polymerase α has been implicated by inhibitor studies (80), but whether DNA polymerase δ is also involved has not been determined. The inhibitor studies have also suggested that DNA polymerase γ might be involved, possibly to carry out the repairlike synthesis at the ends after the terminal hairpin is nicked and the sequence involved has been transferred from the parental to the progeny strand. The covalent attachment of a cellular protein to the 5' terminus of MVM replicative intermediates has also been suggested (34).

A major question concerns the regulation of parvovirus DNA replication as a function of the specific intracellular conditions. For the autonomous parvoviruses to initiate DNA replication, the cell must go through the S phase; hence the notion that control of viral DNA replication might well be comparable to that of cellular DNA synthesis. The AAV story is somewhat different. There is evidence for the existence of a viral system that negatively regulates replication of the AAV genome (82). The model was derived from a study of the replication of a plasmid containing the AAV genome into which had been inserted the SV40 origin of DNA replication. When the hybrid plasmid was transfected into cos 7 cells (monkey cells in which the SV40 T antigen is expressed constitutively), its expected replication was markedly inhibited. The inhibition required a *trans*-active product of the *rep* gene and a *cis*-active target sequence in the terminal repeat of AAV. Therefore, an AAV regulatory circuit was able to inhibit what was effectively an SV40 replicon. Apparently as a consequence of the inhibition of replication, such constructs demonstrated a greatly enhanced propensity to integrate into the cellular genome (Labow, unpublished). By extrapolation it seems likely that the negative regulation of DNA replication observed may also serve to inhibit AAV DNA replication in human cells under nonpermissive conditions. If this proves to be the case, AAV will then have systems to actively inhibit both its own gene expression and DNA replication under nonpermissive conditions. In both instances a product(s) of the *rep* gene has been implicated. Therefore, the notion of AAV as a defective virus must be reconsidered. Rather, if the data described above are indicative, under nonpermissive condi-

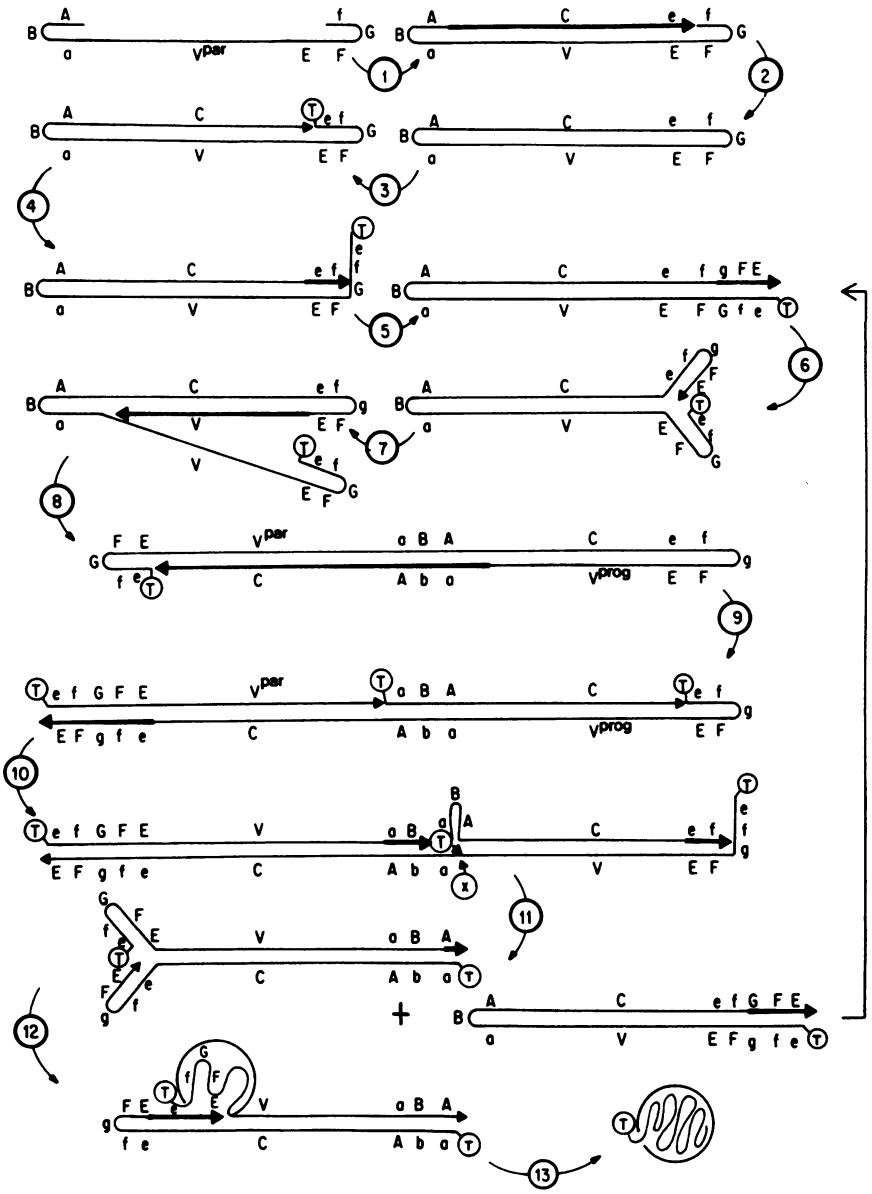

FIG. 3. Model for MVM DNA replication. See text for details. Abbreviations: ABA, 3'-terminal palindrome of virion strand; FGf, 5'-terminal palindrome of virion strand; e, 18- to 26-nucleotide sequence present in replicative intermediates but not in DNA of nuclease-treated virions; T, site-specific nucleases; V, virion strand; V^{par}, parental virion strand; V^{prog}, progeny virion strand; C, complementary strand; X, site of possible topoisomerase action. Revised and reprinted from *Advances in Virus Research* (43a) with permission of the publisher.

tions the autoinhibition of replication might well serve to enhance the probability that the incoming AAV DNA can be integrated into cell DNA to establish a latent infection.

LATENCY

Both AAV and the autonomous parvoviruses can cause cryptic infections in the intact host. Primary lots of cells frequently will yield autonomous viruses after appropriate stimulation or degeneration of the culture. At the level of cell culture it has been difficult to demonstrate integration of the autonomous parvovirus genome. However, the cloned duplex form of the DNA is infectious and can clearly be rescued from the integrated state in the plasmid vector, so that the question of establishing a latent infection by integration still has to be satisfactorily resolved. By contrast, because of the unusual biology of AAV replication, latent infection is readily demonstrable. Initially 20% of lots of primary African green monkey kidney cells and 1 to 2% of primary lots of human embryonic kidney cells were found to be naturally latently infected (69). Berns et al. (18) then demonstrated that the latent infection could be achieved in cell culture by simply infecting continuous lines of human cells with high multiplicities of AAV (250 infectious units per cell) in the absence of adenovirus. Such cultures remained latently infected for >50 passages, and several clones were isolated and characterized. The viral DNA was found to be integrated into cell DNA as a tandem repeat of several copies, with the termini of the viral genome close to the

junction with cellular sequences (33). Although all of the viral sequences were integrated in early passages, free DNA, which was indistinguishable from the duplex form of virion DNA, was present after 100 passages of the clone. Those results were essentially duplicated by several laboratories (63, 86, 95). During the past year, two laboratories have managed to isolate clones from lambda bacteriophage genomic libraries of latently infected cells and to begin characterization at the nucleotide level (81; X. Zhu and R. J. Samulski, personal communication). In one case, three clones containing AAV sequences were isolated from the lambda library (81). Two of the isolated clones represented different junctions between viral and cellular sequences. In each case the terminal repeat was at or near the junction with cell DNA, and all three clones showed evidence of deletions and/or viral sequence rearrangements. A single *Eco*RI fragment of 8.1 kb has been cloned from uninfected cells, mapped, and partially sequenced (81; R. Kotin and K. Berns, unpublished data). About 2.2 kb of cell sequence was inverted and rearranged in the latently infected-cell DNA within the original sequence, and the AAV DNA was inserted 115 bases from one junction of the rearranged sequence with normal DNA. Thus, both viral and cellular sequences were disrupted in the region of the integration. The exact order of occurrence is undetermined. When subclones of either cellular flanking sequence were used as probes of *Bam*HI genomic blots of uninfected-cell DNA, each hybridized to a single band. However, when hybridized to a genomic blot of the latently infected-cell DNA, the flanking-sequence probes lit up either one or two new bands, in addition to the band present in genomic blots of uninfected-cell DNA. The new bands also hybridized to AAV-specific probes. Therefore, it appeared that the latently infected cells contained one original sequence (or more) and at least one copy of sequence that had been disrupted as a consequence of the integration process.

Studies on latently infected cell lines have been carried out by workers in several laboratories, using restriction analysis of genomic blots (33, 86, 95). For every independently derived cell clone, the bands identified as junction fragments between viral and cellular sequences were of different sizes; hence the early conclusion that AAV integrated at random, or at least at a large number of different sites, in the human genome. This conclusion has had to be reassessed because of the evidence for extensive DNA rearrangements found in the sequences from the clone described above. With the availability of probes to assess whether the same normal cell sequences were consistently disrupted, 21 additional, independently derived clones were obtained from three additional laboratories. Of these 21 clones, 14 also showed evidence for disruption of the same normal sequence, and a majority of the new bands resulting from the disruption also contained AAV sequences. It was concluded that AAV integration is highly site specific, within a 7.6-kb sequence that has been mapped, by using genomic blots of mouse-human hybrid cell lines, to human chromosome 19 (81a). By use of biotinylated probes, the unoccupied site has been visualized at 19q13.3-qter (J. Menninger, R. Kotin, D. Ward, and K. Berns, unpublished data).

At high multiplicities of infection (250 infectious units per cell), AAV has been observed to cause latent infections in 10 to 30% of the infected cells (18, 95). The efficiency of the process has led to the development of AAV vector systems for cellular transformation (68, 85, 89, 98, 146, 148). Packaging systems have been developed in which the deleted AAV gene functions are supplied in *trans* from alternative constructs (68, 146). However, even a small amount of sequence overlap between the two constructs leads to sufficient homologous recombination such that there is significant contamination of the vector virion preparation with recombinant wild-type virus. Recently a packaging system has been developed in which there is no possibility of homologous recombination to produce wild-type virus (127). The presence of pure virion vector preparations is essential to determine the role of viral function(s) in the integration process. Without wild-type virus in the preparation, AAV vectors which contain only the original 190 bases from the right end of the genome ligated to both ends of a selectable marker can form latent infections, albeit at an apparently lower frequency than seen with wild-type virus. Some evidence might suggest that without the viral *rep* gene the specificity of integration is lost, but the data are only preliminary.

Rescue of the integrated genome represents the second component of a successful latent infection. As stated above, study of a clone of latently infected cells for more than 100 passages had detected the presence of free copies of AAV only in late-passage cells, as though in some of the cells the integrated sequences could be spontaneously excised from the integrated state. A fundamental question of functional rescue is whether rescue is an inherent part of DNA replication or whether excision occurs first and then DNA replication initiates. The latter possibility is supported by the results of Gottlieb and Muzyczka (57), who isolated from uninfected HeLa cells an enzyme activity that could cleave an AAV DNA clone at or near the junction with cellular sequences. The plasmid substrate was not being replicated. The excision site was not completely sequence specific, and the cutting was stimulated by GC tracts. Support for the notion that rescue might be an inherent consequence of DNA replication has been obtained recently. Replication of an AAV DNA clone into which an SV40 *ori* has been inserted can be achieved in the existing SV40 in vitro DNA replication assay. The assay uses a HeLa cell extract and requires addition of SV40 T antigen. If the AAV-SV40 construct contains the AAV terminal repeats intact, T-antigen-dependent DNA replication leads to the excision of the AAV insert from the pBR322 vector. Both the excised AAV sequences and the resultant pBR322 DNA are covalently cross-linked at their termini by DNA hairpins. Restriction analysis suggests that the AAV is excised in a form that appears to be the putative replicative intermediate illustrated in Fig. 2 after elongation (P. Ward and K. Berns, unpublished data). This structure has now been shown to be specifically processed by the *rep* protein (70a, 136). This excision reaction appears to be much more efficient than the one reported by Gottlieb and Muzyczka (57) and would be in accord with the hypothesis that excision from the integrated state is an inherent part of DNA replication. The potential linkage of replication and excision is also supported by a recent report that the H-1 NS-1 protein is required for both excision and replication (120). The AAV system has proven to be a good model of latency in that it is amenable to detailed molecular analysis. One consequence of these studies has been a better understanding of the process and hence the recognition that integration occurs in a large fraction of cells exposed to virions. This had led to the development of virion vector systems which hold significant promise for gene therapy.

SUPPRESSION OF ONCOGENICITY

The parvoviruses are unique among the nuclear DNA viruses in that they have never been implicated as oncogenic agents. To the contrary, in cases in which the relationships between these viruses and cancer or tumor or transformed cells have been studied, the role of parvoviruses has generally been found to be suppressive. The tentative conclusion from these studies has been that the changes in intracellular milieu in transformed cells which affect the regulation of expression of different classes of cellular genes also affect the expression of viral genes. In turn, viral gene expression may either lead to cell death or inhibit cell division.

Originally, many autonomous parvoviruses were isolated from tumor cells, and so the question of viral oncogenicity did arise (36). However, a variety of studies with intact animals and with cell cultures showed that the presence of these viruses was probably due to the permissive nature of the host tumor cells and that the virus did not cause tumor induction. Most such studies have been done with rodent systems (17, 26, 37, 38, 65, 76, 77, 105, 114, 142–144). Infection in utero is teratogenic, but hamsters which survive such infections with H-1 are significantly less likely than uninfected siblings to develop spontaneous tumors (143). It has been reported that infection of mice by MVM suppressed the growth of Ehrlich ascites tumor cells inoculated intraperitoneally (61). Similar results were obtained with injection of transformed human mammary epithelial cells and H-1 infections in nude mice (52). In culture, transformed mammary epithelial cells were more susceptible to viral killing than were normal mammary epithelial cells. Because cells in culture must pass through the S phase for a productive parvovirus infection to occur, it is not surprising that parvovirus infection is most damaging in utero and in neonatal animals. Adult animals have tissues that contain dividing cells, yet parvovirus infection of most adults is less virulent. This type of distinction has also been found in cell culture; susceptibility to parvovirus infection is dependent not only on cell division, but also on the state of differentiation of the cells.

It is in the sense of possible dedifferentiation that cell transformation may cause an enhanced sensitization to parvovirus infection (53), although transformation per se is not sufficient (106). Two steps in viral replication seem to be involved: the level of NS protein gene expression and DNA replication. In human cell culture systems H-1 virus can replicate in and kill only transformed cells. In one study, cells transformed by and containing viral DNA of human papillomavirus, SV40, adenovirus, hepatitis B virus, Epstein-Barr virus, and human T-cell lymphotropic virus were all susceptible to H-1 killing (53). However, six cell lines that were transformed but no longer contained sequences of the transforming viruses were less susceptible. Only two were fully permissive for H-1, two were semipermissive, and two were resistant. Neither gene expression of the transforming viruses alone in separate assays (e.g., infection of untransformed cells) nor the transformed state alone was sufficient. Apparently an interplay of the two systems was required to achieve an intracellular milieu fully permissive and susceptible to H-1. In another study, human fibroblasts that were morphologically altered and established cell lines as a result of ^{60}Co gamma irradiation, treatment with 4-nitroquinolone-1-oxide, or SV40 infection were more susceptible to MVM infection than were normal, finite, life-span parental strains (39). The level of viral DNA replication was increased 10- to 85-fold. Cell transformation thus correlated with enhanced sensitivity to viral infection and increased viral DNA replication.

The enhanced susceptibility of transformed cells may be due in part to a differential regulation of expression from the p4 promoter which is responsible for transcripts of the NS protein gene (40, 150). Cultures of transformed rat fibroblasts were more susceptible to the cytopathic effects of MVM infection than were their normal homologs. The uptake of virus, viral DNA replication, and structural protein synthesis were comparable in both types of cells; however, the level of NS protein gene expression was significantly greater in the transformed cells. A second step in viral replication was enhanced in the transformed cells because more infectious virions were produced. Another study has also suggested that the level of NS protein synthesis and eventual cellular location of these proteins are significant (122). Two rat cell lines, FR3T3 and NRK, differ in their ability to allow parvovirus DNA replication; viral DNA synthesis is significantly greater in FR3T3 than in NRK cells. Transformation of both cells by the EJ Ha-*ras-1* oncogene renders the FR3T3 derivative, but not the NRK derivative, sensitive to killing by MVM. The transformed FR3T3 derivatives showed enhanced early gene expression, in addition to an already high level of viral DNA replication; the transformed NRK derivatives showed neither enhanced DNA replication nor early-gene expression. Only the transformed FR3T3 derivative showed significant susceptibility to the cytopathic effects of MVM infection, again suggesting that there are at least two steps in viral replication necessary for cell killing and that the two steps appear to be the consequence of different aspects of intracellular regulation (40).

AAV inhibits the replication of adenovirus in a coinfection (28). At least one site of inhibition is the initiation of transcription of the adenovirus early genes (M. A. Labow and K. I. Berns, unpublished data). AAV also inhibits the oncogenicity of adenovirus in intact animals. Adenovirus causes hemorrhagic sarcomas at the site of injection into newborn Syrian hamsters. Addition of AAV to the inoculum lengthens the period and decreases the frequency of tumor induction (78). At the level of cell culture, AAV also inhibits adenovirus transformation (29), although the actual mechanism by which the AAV inhibition works has not been completely defined. One group reported that defective AAV particles which contained only the terminal sequences of the AAV genome could inhibit adenovirus oncogenicity in intact animals (47). Comparable results could be obtained by substituting purified variant genomes with large internal deletions. Therefore, it was hypothesized that some terminal structure of the AAV genome was the functional inhibitor. At the cellular level, AAV was able to prevent transformation by adenovirus (29). It was also possible to inhibit the oncogenicity of adenovirus-transformed hamster cells by infecting the cells with high multiplicities of AAV (108). After the AAV infection, the cells grew at the same rate but to a lower saturation density and they had lost anchorage independence. When the AAV-infected cells were inoculated into newborn Syrian hamsters the induction period was significantly longer and the frequency of tumors was decreased by 70%. The tumors that did arise were only 10^{-3} of the normal volume, but the histology was that of the original hemorrhagic sarcomas. At the molecular level, infection of the transformed cells with AAV did not affect the integrated adenovirus sequences or the specific mRNAs that were detectable in Northern blots. However, the amounts of ^{35}S-labeled E1b 55-kilodalton protein accumulated (the ma-

jor tumor antigen) were decreased by 80%. Therefore, there appeared to be an inhibition at a posttranscriptional level, possibly at the level of translation.

Although the data above suggest the levels at which the inhibition of adenovirus oncogenicity is occurring, they do not define which AAV function is responsible. DNA transfection experiments in cell culture may well bear on the question. In cotransfections of murine cells, cloned AAV DNA was able to inhibit in *trans* >95% transformation to G418 resistance by a plasmid in which the bacterial gene for neomycin resistance was under control of the SV40 early promoter (83). Similar results were seen when the *neo* gene was under control of either the herpes simplex virus type 2 thymidine kinase promoter or the murine metallothionine-inducible promoter. The inhibition was mapped to the AAV *rep* gene. Therefore, a potential second mechanism by which AAV may inhibit adenovirus oncogenicity was found. It is worth noting that the two potential sources of inhibition of oncogenicity, namely, the terminal repeat in *cis* and the *rep* gene in *trans*, are the major sources of overall regulation of the cycle of AAV replication.

Whether AAV naturally functions to repress certain types of tumors is unknown. In addition to the work with adenovirus, suppressive effects by AAV on viral replication, oncogenicity, and transformation by herpes simplex virus (10, 46) SV40 (131), and bovine papillomavirus (66), as well as the oncogene *ras* (75), have also been observed. In an even more general sense, work in several laboratories has demonstrated that exposure of cells to various physical and chemical insults which are sometimes oncogenic serves to activate the AAV genome (152, 153). Such activation might in itself lead to repression of tumorigenesis. At an epidemiologic level, several studies have now found that women with cervical carcinomas are seronegative for antibodies to AAV (56, 94, 138). These data raised the possibility that AAV might actually subserve a protective function.

CONCLUDING COMMENTS

Despite, or perhaps because of, their structural simplicity, a detailed understanding of the mechanisms of regulation of parvovirus replication has been slow in coming. It is now evident that NS proteins are encoded which function *trans* as both positive and negative regulators of gene expression. To date, all the available evidence suggests that the effects of the viral regulatory proteins on nonviral gene expression are entirely negative, whereas the effects on parvovirus gene expression appear more variable, in a manner dependent on the intracellular milieu. Possibly as a consequence of this behavior, the parvoviruses can frequently cause cryptic infections in the intact host, and we have begun to understand (in the case of AAV) the mechanisms involved in latent infection. In contrast to other nuclear DNA viruses that can establish a stable relationship with the host, the parvoviruses are unique in that they have not been associated with oncogenesis in a causal fashion. To the contrary, their presence appears to have a preventive effect. To a large extent this antioncogenic property may be attributable to the specific intracellular milieu required for parvovirus replication, namely, the milieu characteristic of at least some transformed or oncogenic cells, and the generally negative effects of productive parvovirus replication on such cells. The properties of the parvoviruses in model systems raise serious questions about the consequences of human infection. Most adults (85 to 90%) have been exposed to both a human autonomous virus, B19, and AAV. With the exception of aplastic crises in those with hemolytic anemias and nonimmune hydrops of the infected fetus, the negative consequences of such infections are either mild (B19) or inapparent (AAV). In the case of AAV infection there are the unusual consequences (in terms of our usual consideration) of the infection's appearing to be either benign or possibly even beneficial. These are radical possibilities in the framework of mammalian viral infection, and yet we all appreciate that there may be significant numbers of viruses that could behave in this manner which have not yet been discovered, precisely because they do not cause apparent disease. In this sense the parvoviruses represent an excellent model system for continued study.

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