**REVIEW ARTICLE**

**Parvovirus Gene Regulation**

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**INTRODUCTION**

The parvoviruses are among the smallest of the DNA animal viruses (Siegl et al., 1985). The genome is a linear single-stranded DNA of approximately 5 kb which is encapsidated in a naked icosahedral virion 20 to 26 nm in diameter. There are two or three capsid proteins which have overlapping amino acid sequences; the larger species have additional amino acids at the NH₂ termini. The family Paroviridae contains three genera. One, the densoviruses, contains viruses which have been isolated from several species of butterflies and moths. The other two genera, the parvoviruses and the dependoviruses (adeno-associated viruses), contain viruses which infect a broad range of vertebrates. Because our knowledge of the molecular biology of replication is significantly greater for the latter two genera, this review will concentrate on the regulation of gene expression in the parvoviruses and the dependoviruses.

Although both parvoviruses and dependoviruses infect many vertebrate species from birds to man, viruses in the two genera differ significantly in their biology and details of genome structure. Parvoviruses are autonomous in their replication. The major requirement is that a normally permissive cell must undergo the S phase for parvovirus infection to be productive (Tennant et al., 1969; Rhode, 1973; Siegl & Gautschi, 1973; Tattersall, 1972). Parvoviruses are significant pathogens and the kinds of disease that they cause tend to reflect this requirement. Thus, they are major pathogens in utero in several species, and in young animals cause enteritis in dogs, mink and cats, panleukopenia in cats, and aplastic crises in people suffering from haemolytic anaemias such as sickle cell disease (Siegl, 1983a, b, c; Pattison et al., 1981; Serjeant et al., 1981). By contrast the dependoviruses are absolutely defective and require a co-infection with a helper virus for productive infection to occur (Atchison et al., 1965; Melnick et al., 1965; Hoggan et al., 1966; Carter & Laughlin, 1983). Although human infections are common, adeno-associated virus has never been implicated as an aetiological agent (Blacklow et al., 1968 a, b). In the absence of a helper virus the dependovirion penetrates to the cell nucleus where the DNA is uncoated and is then integrated into the cell genome in a highly efficient manner (Handa et al., 1972; Berns et al., 1975; Handa et al., 1977). The genome can be rescued from the integrated state after superinfection of the cell with a helper virus, again in a highly efficient manner (Hoggan et al., 1972; Berns et al., 1975; Handa et al., 1977). In terms of the biological continuity of the dependovirus genome the ability to establish a latent infection in the absence of the helper in some ways compensates for the defectiveness of the virus. Both parvoviruses and dependoviruses commonly cause cryptic infections. The autonomous viruses are often isolated from apparently uninfected cell cultures and up to 20% of the lots of primary African green monkey kidney cells have been reported to be latently infected by dependoviruses (Hoggan et al., 1972; Cukor et al., 1983; Siegl, 1983a). Indeed the dependoviruses were discovered as contaminants of what had been thought to be purified adenovirus (Ad) stocks. Because of the frequent association with Ad the dependoviruses have been popularly called adeno-associated virus (AAV). In recent years it has been demonstrated that, in addition to Ad, herpes simplex virus (HSV) types 1 and 2, can serve as complete helpers for dependovirus replication (Buller et al., 1981). For this reason the parvovirus study group of the International Committee on Taxonomy of Viruses has accepted dependovirus as the official name for the defective paroviruses. However, since AAV is in common use we shall use that designation in this article as well.
Because of the small coding capacity of the parvovirus genome, the virus is clearly extremely dependent on the intracellular milieu for replication. In the case of the autonomous parvoviruses this results in a narrow host range and tissue specificity for individual members of the genus. AAV, on the other hand, has a wide host range. Human AAV serotypes can replicate in cell cultures derived from several different species as long as the cells are co-infected with a helper virus for which the cells are permissive (Hoggan et al., 1966; Cukor et al., 1983). Perhaps this reflects the ability of the helper virus to alter the cell milieu for its own replication. This may explain why viruses totally unrelated to AAV (in terms of nucleotide sequence) can serve as helpers for AAV replication and why the helper viruses can be themselves unrelated. Almost every type of herpesvirus can serve as at least a partial helper for AAV replication and several are complete helpers (Buller et al., 1981). Interestingly, AAV, Ad and herpesviruses represent all the families of linear DNA viruses that replicate in the nuclei of vertebrate cells. Finally, there have been reports that some autonomous parvoviruses isolated from other species can grow in human cells in culture if the cells are co-infected by a human Ad (Ledinko et al., 1969; Tattersall & Ward, 1978).

The aim of this review is to try to bring together the existing data concerning the replication of the various paroviruses. Can we find a common underlying basis for the multiplication of viruses with such different biological properties?

Organization of the genome

AAV DNA strands of each polarity are packaged with equal frequency so that half the particles contain plus strands and the other half minus strands (Rose et al., 1969; Mayor et al., 1969; Berns & Rose, 1970; Berns & Adler, 1972). The autonomous viruses are more variable in their packaging. Minute virus of mice (MVM) and H1 package the minus (antisense) strand 99% of the time, bovine parvovirus the minus strand 75 to 80% of the time, and the human autonomous virus B19 packages both strands equally frequently (Bates et al., 1984; Cotmore & Tattersall, 1984; Siegl et al., 1985). LuIII virus packages only minus strands or both with equal frequency depending on the host cell (Majaniemi et al., 1981; Bates et al., 1984). A major difference between the genomes of the viruses of the two genera has been found in the organization of the terminal sequences. AAV DNA has an inverted terminal repeat of 145 bases (Gerry et al., 1973; Kozcot et al., 1973; Berns & Kelly, 1974; Fife et al., 1977; Lusby et al., 1980). The terminal 125 bases are palindromic, but the overall palindrome is interrupted by two shorter palindromes symmetrically disposed on either side of the centre of the larger palindrome. As a consequence, when the terminal 125 bases are folded to optimize potential base pairing, a T-shaped structure is formed which is thought to serve as a primer for DNA replication (Straus et al., 1976b; Berns & Hauswirth, 1983). The structure has been shown to be important in AAV DNA replication in a manner that transcends, at least partially, the primary sequence. Part of the sequence of the short internal palindromes can be substituted; so long as the conformation is retained the mutants are viable (LeFebvre et al., 1984). Both termini of the autonomous virus genome are also palindromic, but the sequences are unrelated to one another (Bourguignon et al., 1976; Astell et al., 1979a, b; Salzman & Fabisch, 1979; Rhode & Klaassen, 1982). The palindrome at the 3' end of the minus or usual virion strand is about 120 bases long and is again interrupted by two small palindromes about the centre, so that a T-shaped structure similar to that of AAV DNA is formed when the overall palindrome is folded on itself. The palindromic sequence at the other end (5') of the autonomous virus genome is longer (160 to 200 bases) and normally different in sequence. However, the terminal sequences of the human B19 virus are repeated (Shade et al., 1986). Unfortunately these sequences are longer and have not yet been cloned intact. In any event the sequences at the termini in the various viruses suggest both a continuous spectrum between AAV and the autonomous viruses and the common structure at the 3' end demonstrates a close relatedness between the two genera. The relationship is made even more evident when the overall sequences of the genomes are compared.

The complete nucleotide sequences of AAV2 and of several of the autonomous virus genomes have been determined (Lusby & Berns, 1982; Srivastava et al., 1983; Rhode & Paradiso, 1983; Astell et al., 1984; Shade et al., 1986). The basic organization is the same for all of them. In every
Fig. 1. (a) Schematic presentation of the genetic organization of the genome of MVM, a member of the genus *Parvovirus* [modified after Cotmore *et al.* (1983)]. (b) Schematic presentation of the genetic organization of AAV2, a member of genus *Dependovirus* [modified after Srivastava *et al.* (1983) and Carter *et al.* (1984). Reprinted with permission from Siegl *et al.* (1985)]. The sizes of mRNAs are indicated in kb.

case there are two large non-overlapping open reading frames (ORF), each occupying most of one half of the genome (Fig. 1). As detailed below, the left side ORF is involved in regulatory functions and the right side codes for structural proteins. There is no detectable nucleotide sequence homology among AAV and the various autonomous virus genomes by hybridization and little, if any, among autonomous viruses isolated from different species, with the exception of feline panleukopenia virus (FPV) and the two viruses thought to be recently derived from FPV, mink enteritis virus and canine parvovirus. Yet recent analyses by Shade *et al.* (1986) have demonstrated that there are significant homologies between the human B19 virus, MVM and AAV, both in the regulatory regions on the left side of the genome and even more surprisingly perhaps, also in the right side ORF coding for coat proteins. Interestingly, the degree of homology between the B19 genome and the AAV genome is slightly greater than that observed between B19 and MVM. It is this sort of data which confirms the true relatedness of the
parvovirus and dependovirus genera and makes even more intriguing the apparent biological
differences in their requirements for replication and host ranges. Numerous regulatory signal
sequences have been mapped on the various genomes. These will be detailed in the next section.

Transcription

Generalized transcription maps for AAV and the autonomous viruses are shown in Fig. 1. For
the latter (MVM), two promoters have been identified at map positions (mp) 4 and 38 (Pintel et al., 1983; Carter et al., 1983). Three major transcripts have been noted. Two begin downstream
from the p4 promoter. Both contain a small potential intron between mp 46 and 48 and one has a
second larger spliced region from mp 10 to 40. The third transcript begins at mp 38 and also
contains the smaller intron noted above. All three terminate at mp 95 and are capped at the 5’
terminus and polyadenylated at the 3’ end.

The AAV transcription map is quite similar. There are promoters at mp 5 and 40 also, but
there is an additional promoter at mp 19 (Green & Roeder, 1980b; Lusby & Berns, 1982; Carter et al., 1983). At least one transcript has been noted to start at each of the promoters and all are
spliced to a variable extent at an intron between mp 41 and 46 (Laughlin et al., 1979; Green &
Roeder, 1980a; Green et al., 1980; Lusby & Berns, 1982; Srivastava et al., 1983). All three
transcripts terminate at mp 96 and are capped and polyadenylated. Again, B19 appears more
like AAV. Sequence analysis has suggested the possibility of a promoter near mp 19 in B19
(Shade et al., 1986). In all cases, both defective and autonomous, the major RNA species isolated
from the cytoplasm of infected cells late in infection is the spliced species of the RNA initiated at
mp 38 to 40. The species is translated to the major coat protein of the virion (Jay et al., 1981). In
the case of AAV a variety of experiments have suggested that the p40 RNA may be alternatively
spliced to encode the ORF for the largest coat protein VP-1 (Hermonat et al., 1984).

Proteins

The AAV2 virion is composed of three structural proteins, VP-1 (92000 mol. wt., 92K), VP-2
(72K) and VP-3 (65K) (Johnson et al., 1971, 1975; Rose et al., 1971; Salo & Mayor, 1977). The
third represents about 80% of the total mass. Experiments with in vitro translation have
demonstrated that all three are translated from a spliced p40 transcript (Jay et al., 1981). A
problem arises with this conclusion; in the spliced p40 RNA illustrated in Fig. 1 the first AUG
occurs in phase in the ORF at a position which would permit synthesis of a protein whose
maximum size could equal that of only VP-3. The seeming paradox is apparently resolved in two
ways. VP-2 has been reported to initiate translation at an ACG upstream from the first potential
AUG (Becerra et al., 1985). VP-1 translation may require a second splice of the p40 transcript,
either in addition to or in place of that shown in Fig. 1 (Hermonat et al., 1984).

The three structural proteins of the autonomous virus capsid are similar in size to those of
AAV; however, the relative amounts of VP-2 and VP-3 are variable depending on the host cell
and the time during infection (Johnson, 1983). Conversion of VP-2 to VP-3 by proteolytic
digestion of intact particles has also been reported (Tattersall et al., 1977).

In the last several years evidence has accumulated for the existence of non-structural (NS)
proteins. The first evidence of a NS protein was in the case of MVM where sera from an infected
animal could detect a specific polypeptide of 83K (Cotmore et al., 1983). This protein
corresponds to the left side ORF and is phosphorylated. Similar proteins have been reported for
several more autonomous viruses. A second smaller NS protein of 24K has also been reported for
the autonomous parvoviruses (Cotmore et al., 1983). Because of the requirement for a helper
virus, it has been much more difficult to demonstrate non-structural proteins during AAV
infection. Polypeptides have been synthesized using p5 and p19 transcripts in in vitro translation
systems. More recently peptides that corresponded to sequence in the AAV left side ORF were
synthesized, coupled to carrier proteins, and the conjugates used to immunize rabbits. The
antisera raised can immunoprecipitate proteins of 70K and 44K specifically from cells
productively infected by AAV and Ad (Mendelson et al., 1986). Proteins corresponding to
translation products of both the spliced and unspliced species of the p5 and p19 transcripts have
been detected.
One additional protein has been noted in the case of the autonomous viruses. The replicative form (RF) DNA has a protein of approximately 60K covalently linked to the 5' terminus (Revie et al., 1979). The origin of the protein, as well as its specific function, is not yet certain. However, unpublished data (J. Bodnar & D. Ward, personal communication) would suggest that the protein may be of host cell origin (by DNA sequence analysis and immunological assay). If one of the functions of the protein is to cleave hairpin structures in RF DNA, then the protein may correspond to an activity described by Gotlieb & Muzyczka (personal communication) which is found in uninfected cells and cleaves the junction between AAV and pBR322 sequences in recombinant plasmids.

**Milieu requirements for replication**

Although all viruses are defined as obligate intracellular parasites, the Parvoviridae are more dependent than most on the intracellular milieu for their replication. This is undoubtedly the consequence of the very small size of their genomes. The obvious corollary of this point is that their heavy dependence on cell function makes them potentially very good probes of cell function. The autonomous viruses have an absolute dependence on the cell cycle. Some function(s) that occurs in the S phase is required for the conversion of the double-stranded RF to mature single-stranded virion genomes (Tennant et al., 1969; Siegl & Gautschi, 1973). In studies of the tissue specificity of different strains of MVM a specific requirement for a cell function has been suggested for the conversion of the parental genome to the RF (i.e. this step does not occur in the non-permissive cell) (J. Tal, personal communication). Of course the possibility also exists that the failure of the step is the consequence of an inhibitor rather than the lack of a requirement.

The close relatedness of AAV to the autonomous parvoviruses and the lack of extensive sequence homology between AAV and either of the helper viruses might lead one to suppose that there is some single critical function supplied by the helper. This notion turns out to be naive. In fact it has been possible to demonstrate that all aspects of AAV macromolecular synthesis are affected by the helper. Because the genetics of Ad have been understood in more detail for a longer time than those of HSV the specificity of the Ad helper functions are much better known. Ad early region E1A which is required for optimal transcription of the rest of the Ad early genes is also required for AAV transcription (Richardson & Westphal, 1981; Janik et al., 1981; Laughlin et al., 1982; Tratschin et al., 1984b). In a manner as yet not understood Ad E1B can substitute for E1A (Richardson & Westphal, 1984). E1B has also been suggested to be required for AAV DNA replication and for rescue of the AAV genome from the integrated state (Ostrove & Berns, 1980; Laughlin et al., 1982). Ad E4 is also required for AAV DNA replication (Richardson & Westphal, 1981). One of the several E4-coded polypeptides forms a complex with the 55K T-antigen encoded in E1B during lytic infection (Sarnow et al., 1984). It is this polypeptide that seems to be required for AAV DNA synthesis (R. J. Samulski & T. Shenk, personal communication). The Ad VA RNAs are required for synthesis of AAV structural proteins (Janik et al., 1982). Finally, Ad E2A (which codes for a 72K single-stranded DNA-binding protein) has been suggested to be involved in one or all of the following AAV functions: DNA replication, transport of mRNA from nucleus to cytoplasm, and protein synthesis (Handa et al., 1975; Jay et al., 1981; McPherson et al., 1982). This region clearly affects AAV replication, but, as suggested above, the exact mechanism is currently a matter of some controversy. It seems likely that HSV as a helper virus must also be able to regulate the synthesis of the various AAV-specific macromolecules. If the helper virus gene products that are required for AAV replication were to act directly on the AAV genome or transcripts, it would again raise the problem of the lack of significant discernible homology among the three viruses. If, on the other hand, the helper virus functions are required to modulate the intracellular milieu so that AAV replication can occur, then the lack of sequence homology is not so much of a problem. The latter interpretation would also fit well with the observations of Richardson & Westphal (1981) that Ad E4 transcripts alone are sufficient to help AAV replication in Vero monkey cells; yet in other cell lines Ad E1A function is required specifically for AAV transcription to occur.
Both of the helper viruses for AAV replication code for their own DNA polymerases. Yet it seems likely that AAV uses the cellular DNA polymerase alpha, at least with Ad as the helper. Ad E2B mutants in the DNA polymerase are negative for Ad DNA synthesis but support AAV DNA replication (Straus et al., 1976a). Therefore, this aspect of AAV replication is comparable to that of the autonomous viruses which have also been reported to use DNA polymerase alpha for DNA replication. In a similar manner the cellular RNA polymerase II is used for transcription of the genomes of paroviruses of both genera (Carter et al., 1983). In the case of both viruses the role of the regulatory genes may be to create the milieu in which these polymerases and other cellular enzymes can be used for viral replication.

**Genetic studies**

The intact duplex form of the AAV genome has been inserted into bacterial plasmids (Laughlin et al., 1983; Samulski et al., 1982). Such clones are biologically active; when the recombinant plasmid is transfected into human cells in culture that have been co-infected with Ad, the AAV genome is rescued from the plasmid, replicates and infectious virions are produced. Because the sequence of the entire AAV genome is known, it is possible to make specific mutations at any point, amplify the altered genome in Escherichia coli and then to determine directly the mutant phenotype in human cells. The relative simplicity of this system has permitted a detailed genetic analysis of the AAV genome. Nonsense mutations, insertions or deletions anywhere within the ORF on the left side of the genome block DNA replication (Hermonat et al., 1984; Tratschin et al., 1984a). Such mutants can be complemented in trans. Therefore, AAV encodes one or more gene products in this region which are required for DNA replication. Deletions of sequences within the inverted terminal repeats of the genome which cannot self-repair also block DNA synthesis (Samulski et al., 1983; Senapathy et al., 1984). Such mutants cannot be complemented. The cis-active nature of the terminal repeat sequences supports the notion that they function as the origin (ori) for DNA replication. Mutations with the ORF on the right side of the genome do not block DNA replication; thus, the coat proteins are not required for DNA synthesis (i.e. there is not a push–pull type of synthesis wherein the coat proteins remove one strand from the template so that another may be laid down). However, accumulation of single strands does require coat protein synthesis; apparently sequestration into capsids is required for accumulation.

The same types of study have been done with the autonomous paroviruses. Again the duplex form of the genome cloned into bacterial plasmids is biologically active, but the efficiency of rescue is not as great as that seen with AAV so that the experiments are somewhat more difficult (Merchlinsky et al., 1983). However, the results are equivalent. The ends are needed in cis for DNA replication and the left side ORF must be intact, but can also be complemented in trans. Again the right side ORF appears to be needed primarily for sequestration of mature virion single strands.

**Regulation of gene expression**

The genetic studies described above demonstrated that one or more of the proteins encoded in the left side of the parovirus genome were required for viral DNA replication. Similar studies have also shown that one or more of the proteins from the same region also serve to regulate viral gene expression. Rhode (1985) has reported that the H1 virus 76K non-structural protein greatly enhances expression from the right side p38 promoter. The normal transcript from the p38 promoter covers the right side ORF and encodes the major capsid proteins. In the reported experiments the p38 promoter was placed upstream from the bacterial chloramphenicol acetyltransferase (CAT) gene and the recombinant plasmid was transfected into cells in culture. When transfected alone the p38 CAT construct expressed CAT activity only weakly, but when co-transfected with a second construct expressing the 76K non-structural protein or into cells that were infected with intact H1 virus, the CAT expression was greatly increased. From these results Rhode concluded that the 76K protein could transactivate expression from the p38 promoter.
Similar, but somewhat more detailed, experiments have been possible using AAV. These experiments have used mutagenized clones of the duplex form of the AAV genome inserted into pBR322. The mutated clones were transfected into human cells in culture that had been infected by Ad and the accumulation of AAV transcripts was detected by Northern blotting (Labow et al., 1986). Insertion of 8 base sequences (frameshift mutations) anywhere in the left side ORF caused an over 90% decrease in the accumulation of transcripts from all three of the AAV promoters (p5, p19 and p40). This decrease was observed in both ori- and ori+ constructs. The lack of transcripts in the mutants could have been caused either by decreased transcription or by decreased transcript stability. Rates of transcription were measured by radioactive pulse-labeling of isolated nuclei from AAV-transfected cells. The frameshift mutants were clearly defective in transcription, so that at least part, if not all, of the decrease in accumulation of AAV transcripts noted was due to this factor. The left side ORF frameshift mutants could be complemented in trans; with complementation accumulation of transcripts from all three promoters was increased significantly. Complementation was observed for both ori- and ori+ mutant constructs. Thus, one or more products of the ORF on the left side of the AAV genome activates in trans transcription from all three of the AAV promoters.

A frameshift mutant at mp 42 on the extreme right end of the left side ORF had a particularly interesting phenotype. This mutant was severely defective for DNA replication: less than 1% of the normal amount of AAV DNA was synthesized. Yet under the same conditions transcript accumulation was only depressed by five- to tenfold. These data suggest that there are separate AAV functions in the left side ORF for activation of transcription and DNA replication, possibly residing primarily in different domains of one or both of the protein(s) encoded in the ORF.

Additional experiments have suggested that the AAV genome has the capability of down-regulating its own transcription, as well as positively regulating it. The p40 promoter has been reported to function in human cells when the upstream portion of the AAV genome is not present. In the series of experiments described above a deletion mutant missing sequences from mp 3 to 23 (including both the p5 and p19 promoters) accumulated intermediate levels of p40 transcripts. A second type of cis-active negative regulation has also been observed. Normally the p40 transcripts represent about 90% of the total AAV RNA. But when a deletion between mp 10 to 37 was complemented, the p5 transcript level was about tenfold higher than normal and equal to the complemented level of p40 transcript accumulation (which was normal). The level of p5 transcript accumulation from the complementing genome was at the normal level. This then would appear to be an example of cis-active negative regulation. We also know that bringing the p5 promoter into proximity with sequences around the p40 promoter is not a factor in the increased accumulation of the transcripts, because deleting all the genome sequences between mp 15 to 85 still leads to high levels of the p5 transcript upon complementation.

Two possible mechanisms by which the mp 10 to 37 sequence might lead to a decrease in accumulation of transcripts from an upstream promoter include inhibition of initiation or elongation of transcription. From nuclear run-on experiments, initiation at p5 and p19 actually appears to be equivalent to that at p40. Therefore, it would seem more likely that the negative regulation results from inhibition of elongation or attenuation. Attenuation as a mechanism of regulation of transcription has been observed by Aloni and his colleagues in the case of MVM (Ben-Asher & Aloni, 1984). Another example of negative self-regulation is the observation by Tratschin et al. (1986) that in Ad-transformed human cells (293 cells) the AAV left side gene products appear to inhibit p40-driven gene expression in trans.

It would thus appear that both the autonomous parvoviruses and the dependoviruses not only code for structural proteins and proteins required for DNA replication, but also positively and negatively regulate the expression of their own genomes. Thus at the extreme end of the spectrum, AAV gene expression is regulated by an interplay of factors from three genomes, those of the cell, the helper virus and that of AAV itself. It is clear that AAV gene expression is a consequence of a dynamic equilibrium involving the effects of the three genomes. However, to this point we have only considered the effects of the three genomes on AAV gene expression. We have not considered the consequences of AAV gene expression on either helper virus or cellular
gene expression. In fact there are significant effects on both cellular and helper virus expression which help to make clearer the complex regulatory interplay involved in productive viral infection.

Inhibition of the helper by AAV

Co-infection of AAV with Ad results in a marked inhibition of Ad replication (Carter et al., 1979). If AAV infection is delayed until Ad DNA replication has started, Ad replication is not detectably affected even though AAV replication is normal (Blacklow et al., 1967; Parks et al., 1968). The temporal nature of the inhibition would suggest two possibilities. The first is that prior to the onset of Ad DNA replication, there is competition between Ad and AAV for some common factor that is in limited supply. Thus, the balance between the numbers of the two types of genome would be critical. If there were too many AAV genomes the functioning of the Ad genome might be repressed to the point where it could no longer function as a helper for AAV replication: i.e. AAV could be in effect autoinhibitory. Indeed this has been observed (Carter et al., 1979): too high an AAV multiplicity of infection causes an inhibition of AAV replication. The second possibility is a corollary of the temporal nature of the inhibition, namely that AAV infection inhibits Ad DNA replication. The inhibition could result directly from competition for a limited transcriptional apparatus or some AAV gene product(s) might directly inhibit Ad early gene expression. Preliminary nuclear run-on experiments involving pulse-labeling of nuclei from cells co-infected with Ad and AAV reveal that there is extensive AAV transcription but barely detectable amounts of early Ad gene expression (clearly there was enough to help AAV). These results were undoubtedly exaggerated by the fact that AAV DNA replication had occurred. To measure the effect on Ad gene expression directly it will be essential to avoid a dosage effect. Normally AAV does not appear to inhibit HSV replication. This may simply be a reflection of the very lytic nature of HSV infection. Virion AAV has been reported to inhibit the replication of helper herpesvirus of turkeys in chicken fibroblasts (Bauer & Monreal, 1986).

AAV can also inhibit the oncogenic properties of both HSV and Ad in model systems. AAV infection of HSV-transformed hamster cells significantly inhibited the number of tumours caused and completely prevented metastasis (Cukor et al., 1975; Blacklow et al., 1978). AAV co-infection inhibited Ad tumourigenesis in neonatal Syrian hamsters and prevented Ad transformation of cells in culture (Kirchstein et al., 1968; Mayor et al., 1973). As was the case with HSV cited above, AAV infection of Ad5-transformed hamster cells reduced the frequency of tumour formation after inoculation of newborn animals by 80%, increased the time for tumour induction, and decreased tumour volume by 1000-fold (Östrove et al., 1981). The AAV-infected transformed cells had a normal growth rate but a reduced capacity for cloning in methyl cellulose (i.e. were more anchorage-dependent). At the molecular level the striking difference was an 80% decrease in the amount of the major 58K T-antigen present per cell. Interestingly, increasing the cell inoculum by tenfold restored the original rate of tumour formation, time for induction, and tumour size. Thus, the inhibition resulted in quantitative changes in the transformed phenotype.

The autonomous parvoviruses have also been reported to be oncolytic. The most likely explanation for this phenomenon has been simply that the tumour cells divide and thus are permissive for the autonomous parvovirus replication with a resultant cytopathic effect. However, inhibition of specific gene expression in the tumour cells has not been investigated.

The general notion that the product(s) of the left side rep gene enhance AAV transcription but are inhibitory for Ad gene expression is dependent in part on active Ad co-infection, at least with respect to the activation of AAV gene expression. As discussed above, Tratschin et al. (1986) have recently reported that upon transfection of HeLa cells using CAT constructs in which the bacterial gene for CAT is under control of the AAV p40 promoter, the products of the AAV rep gene enhance CAT expression. Without Ad co-infection the effect was marginal; with Ad co-infection a significant enhancement was observed. However, a very different picture was observed when human 293 cells were transfected with the same CAT constructs. Human 293 cells are Ad-transformed and express only Ad E1A and E1B products. In the absence of Ad co-infection the presence of an intact AAV rep gene inhibited CAT expression. Co-infection with Ad reversed the results: now AAV rep gene expression enhanced CAT expression from the AAV
p40 promoter. These results begin to suggest the complexity of the interaction at the regulatory level between AAV and Ad gene products. First, Ad E1A appears to enhance AAV rep gene expression which (i) seems in turn to inhibit both E1A and E1B expression and (ii) may also inhibit AAV p40-controlled expression when the only Ad gene expression is from E1A and E1B. Second, expression from additional Ad genes changes the effect of the AAV rep gene products on p40-controlled expression from being negative to positive but does not apparently alter the negative effects on Ad E1A and E1B expression. Further evidence for this type of back and forth regulation of the levels and effects of AAV upon gene expression are described below.

Additional inhibitory effects of the AAV rep gene products

The ability of the AAV rep gene product(s) to negatively regulate Ad gene expression and even its own p40-controlled expression has been described above. More general inhibitory properties of the AAV rep gene products have also been observed. When the bacterial gene for resistance to neomycin is cloned into pBR322 under control of the early promoter of simian virus 40 (SV40), the resulting recombinant plasmid pGCcos3neo can transform murine cells in culture to resistance to the drug G418 (Graf et al., 1984). Co-transfection of the murine cells with a plasmid containing the intact AAV genome plus pGCcos3neo can reduce the number of transformed colonies by over 99% when the ratio of AAV plasmid to pGCcos3neo is 50:1. The effect is dose-dependent, but even at a ratio of 1:1 at a number of DNA concentrations, greater than 90% inhibition is observed.

In order to exhibit the inhibitory properties the AAV insert does not need to be potentially able to replicate, as ori- mutants are still inhibitory. Similarly, major deletions of the right side of the AAV genome have no effect on the inhibition of the permanent transformation to neomycin resistance. Only mutations of the AAV left side ORF (rep gene) abolish the inhibitory effect (an 8 base frameshift insertion at mp 32 abolishes the inhibitory effect). This would suggest that a functional AAV gene product(s) is required for the inhibitory effect since the mutation removes no AAV sequences. Interestingly, a similar insertion mutation at mp 11 has much less effect on inhibition. This would indicate that a major effector of the inhibition is the 44K protein encoded by the p19 transcript. This would be the first instance where it is possible to ascribe a specific phenotype to the 44K protein. (Remember that both the 70K and the 44K proteins are translated from the same reading frame and that at least the 70K protein is needed for AAV DNA replication and transcription.)

The inhibitory effect on permanent transformation is not limited to pGCcos3neo. It is also observed in three additional situations involving transformation of murine cells. (i) The neomycin resistance gene can be replaced by the E. coli gpt (guanylphosphotransferase) gene. (ii) The SV40 early promoter can be replaced by the murine metallothionein-inducible promoter. (iii) Permanent transformation by a plasmid containing the HSV thymidine kinase gene under control of its own promoter is also inhibited (M. A. Labow, L. H. Graf & K. I. Berns, unpublished data). This result is of particular note since this gene was the first to be used to demonstrate permanent transformation of a mammalian cell after DNA transfection.

At least three explanations are possible to account for the ability of the AAV rep gene to inhibit permanent transformation in the manner described. Expression of rep may be lethal to the transfected cell. Although AAV DNA replication may be lethal for the cell as described below, we do not believe that AAV rep gene expression per se is lethal. AAV infection of Ad5-transformed hamster cells inhibits Ad gene expression but does not affect cell viability or growth rate (Ostrove et al., 1981). Similarly, intact AAV can latently infect continuous lines of human cells with high efficiency (Hoggan et al., 1972; Handa et al., 1977). Finally, in experiments to be described below, under the appropriate conditions the cells can be transformed to neomycin resistance in a co-transfection with an intact AAV rep gene.

A second possibility is that the AAV rep gene product prevents integration of the selectable gene into the cell genome. We also believe this to be unlikely. Integration of AAV DNA itself in the establishment of latency is not inhibited nor is the integration of a selectable marker itself under appropriate conditions. However, this possibility has not been rigorously excluded in a general way.
A third possibility is that the inhibitory effect is exerted at the level of gene expression. To test this possibility experiments have been done in which the transient expression of CAT activity has been measured when CAT constructs under control of the SV40 early promoter have been co-transfected with AAV plasmids. Transient expression of CAT activity is also blocked by the presence of a functional AAV rep gene (M. A. Labow, L. H. Graf & K. I. Berns, unpublished observations; Tratschin et al., 1986). Therefore, it seems likely that the major inhibitory effect on permanent transformation seen when AAV plasmids are co-transfected with selectable markers on separate plasmids is also at the level of inhibition of gene expression.

It was noted earlier that Tratschin et al. (1986) had found that the regulatory effects of the AAV rep gene products were different when only the Ad E1A and E1B products were present in the cell compared to when there was a productive Ad infection. Among the additional Ad genes required for AAV replication is E4. One of the E4 products has been reported to form a complex with the major E1B 55K protein. The addition of a plasmid that expresses Ad E4 to a co-transfection of murine cells by pGCcos3neo and an AAV plasmid blocks the AAV inhibitory effect and permanent transformation occurs, approaching at times the levels observed after transfection by pGCcos3neo alone. Similarly, an at least partial reversal of the AAV inhibitory effects was seen in transient expression assays with CAT constructs when the Ad E4 expression plasmid was added to the transfection mixtures. Again the complex matrix of regulatory interactions between specific Ad and AAV genes can be appreciated.

Concluding remarks

We can now return to the original question raised at the beginning of this review: which is the better indication of the relatedness of the autonomous and defective parvoviruses, the obvious structural and genome organization similarities or the significant differences in biological properties? The requirements of the autonomous viruses for a cell to go through the S phase and the rather narrow host- and tissue-specificity have made clear their need for an appropriate intracellular milieu. What has become more evident in very recent times is that the multiple functions necessary for AAV replication that are supplied by the helper virus are likely in large part also to serve to change the intracellular milieu so that AAV gene expression and replication can occur. If that notion is correct and no specific helper virus proteins are needed to interact directly with the AAV genome and/or gene products, then it ought to be possible to find a cell which already provides a suitable milieu for AAV replication in the absence of a helper virus. To a large extent this has been done. Both Ad-transformed hamster and human cells will support AAV transcription and heating HeLa cells to 42 °C significantly enhances AAV transcription in the absence of the helper virus. Even more interesting has been the discovery by zur Hausen and colleagues that Chinese hamster embryo cells that have been transformed by SV40, when treated with certain carcinogens or grown in low concentrations (1 μg/ml) of cycloheximide will support AAV DNA replication (Schlehofer et al., 1983). Much of this work has been independently confirmed by E. Winocour (personal communication).

The rapid pace of discovery serves to support the original notion that parvoviruses are good subjects for study because of their small size and close dependence on cellular function. The study of dependoviruses is complicated because of the need for the helper virus. On the other hand all viral infections represent interactions between the virus and cell genomes. Usually only one side is amenable to detailed analysis in context because of the complexity of the cell genome. In the case of the dependoviruses there is the eternal triangle, which affords the opportunity to study the interactions between two of the characters fairly readily and in detail.

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