Defective viral vectors as agents for gene transfer in the nervous system

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Abstract

Viral vectors have attracted great interest as vehicles for gene therapy. Due to concerns regarding continued viral gene expression in several systems, new approaches have been sought for gene transfer in the nervous system. This article reviews the general concepts and basic biology of defective viral vectors. These are vectors which can package into a viral coat but contain no viral genes, thereby allowing efficient gene transfer in the absence of viral gene expression in target cells. The defective herpes simplex virus (HSV) vector has been applied to numerous interesting questions in neurobiology. The inability to completely eliminate helper viruses has raised concern regarding the application of this vector to human disease. The adeno-associated virus (AAV) vector has recently been introduced into the nervous system. This vector harbors no viral genes, however helper viruses can also be completely eliminated from the system. Although the smaller size may limit the range of applications for this vector, it has received great interest as a potential agent for gene therapy in the nervous system. Potential future directions are discussed as well.

Keywords: Defective viral vectors; Gene transfer; Nervous system

1. Introduction

Viral vectors have gained widespread use as vehicles for somatic gene transfer into the central nervous system (CNS). The proliferation of publications, meeting presentations and reviews such as those represented in this volume attest to the degree of interest in and acceptance of this technique by the neurobiology community. In order to genetically modify cells of the central nervous system *in situ*, DNA viruses are used as the basis for currently available vectors. Although several DNA viruses have been modified or exploited in a variety of ways, viral vectors can in general be assigned to one of two major categories: recombinant viral vectors or defective viral vectors. Recombinant vectors have been the most widely used vehicles in neurobiology and have been described in great detail elsewhere in this issue. This group will be discussed briefly below solely for the purposes of definition. Defective viral vectors have received more limited use. However, significant advancements and achievements have been made using these systems. This article will review the basic biology of defective viral vectors and

certain specific applications will also be presented for illustrative purposes.

Recombinant viral vectors can be viewed as any virus which contains a foreign gene inserted within a variant of the native viral chromosome. Adenovirus (Ad) (Berkner, 1988; Graham and Prevec, 1992; Goff and Shenk, 1993; Kozarsky and Wilson, 1993) and herpes simplex virus (HSV) (Roizman and Jenkins, 1985; Shi et al., 1984) have been the principle DNA viruses used to create recombinant vectors, and they are discussed in detail elsewhere. The term recombinant vectors stems from the fact that the gene of interest is most often inserted within the viral genome by homologous recombination. Since wild-type viruses usually possess some degree of pathogenicity, these viruses are then further modified in order to reduce the virulence. Thus one or more genes essential for viral replication may be removed by homologous recombination in the same manner that the foreign gene is inserted. The foreign gene may even be inserted in place of some gene essential for viral pathogenicity, thereby achieving both goals with a single recombination event. Usually the gene(s) removed are essential for viral replication, which is one of the major causes of virus-associated pathology (Engelhardt et al., 1994; Halbert et al., 1985). Vectors are then produced and grown in tissue culture using cell-lines which constitutively express the missing viral gene product, so that a

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productive infection can occur within these cultured cells but will not occur *in vivo* (DeLuca et al., 1985; Shenk and Williams, 1984). Still other deletions have been generated which would serve to limit neurovirulence or prevent inflammatory responses to the continued expression of viral gene products within the target cells (Ginsberg et al., 1991; Simon et al., 1993; Zabner et al., 1994). Among practitioners of gene transfer, these vectors have been variously described as reduced virulence, replication-deficient, replication-defective or defective. Since these vectors are generated by insertion or deletion of genes via homologous recombination, for the purposes of the current discussion the term recombinant viral vectors will be used to describe this class of reagents.

2. Defective viral vectors

Defective viral vectors represent the other major group of DNA viral vectors. The major difference between these two groups is that defective vectors do not possess any viral genes but contain only recognition signals which permit replication and packaging of any foreign DNA into a viral coat (Spaete and Frenkel, 1982). DNA viruses of all varieties possess signals which permit recognition of the DNA genome by viral proteins in order to initiate replication as well as to permit packaging of the progeny DNA into new viral particles (Frenkel et al., 1980; Vlazny and Frenkel, 1981; Stow and McMonagle, 1982; Spaete and Frenkel, 1985; Deiss et al., 1986). These recognition signals do not encode any viral gene products and therefore they are by definition not genes. These have been called '*cis-acting*' signals or elements because, in the presence of viral proteins, they can only allow replication and packaging of the DNA sequences to which they are covalently attached. The necessary viral proteins are then said to function '*in trans*' because they can promote replication and packaging of any DNA existing within the same nuclear compartment and which bears the appropriate *cis-acting* elements. The genome of defective viral vectors thus contain only viral *cis-acting* elements which permit DNA replication and packaging into a viral coat in the presence of the appropriate *trans-acting* factors.

The term defective viral vectors derives from a variant observed in nature called the defective interfering particle (DIP) (Von Magnus, 1954; Huang, 1973; Frenkel, 1981). Often when viruses replicate mutations occur which serve as part of the basis for natural selection. Certain viruses are highly recombinogenic, however, and therefore at some frequency will generate replicative products which harbor substantial deletions in the viral genome. HSV is an example of such a virus (Frenkel et al., 1976; Locker and Frenkel, 1979; Frenkel et al., 1980; Locker et al., 1982). When grown at a high multiplicity of infection (the infectious virus-to-cell ratio), particles will be generated which contain only a limited subset of the 150 kb HSV genome

(Frenkel et al., 1976). These particles contain deletions of numerous essential viral genes, and therefore cannot replicate on their own (Frenkel, 1981). Thus they are 'defective' with respect to replication. These deleted genomes retain the necessary *cis-acting* elements, however, and therefore can package into an HSV coat (Spaete and Frenkel, 1982; Stow and McMonagle, 1982). The missing viral proteins are then provided by wild-type HSV which will co-infect the target cell along with the DIP (Kwong and Frenkel, 1995). Thus the DIP cannot autonomously replicate but absolutely requires co-infection of a cell by this 'helper virus' for propagation. Since DIP can also actively interfere with replication of the helper virus, the term defective interfering particle is used to describe these natural variants (Frenkel et al., 1980; Frenkel, 1981).

Defective viral vectors represent a synthetic variant of this phenomenon. Rather than depend upon nature to generate the necessary vehicle, modern molecular biological techniques have been used to isolate the appropriate viral *cis-acting* elements and splice them together with any interesting foreign gene. Most frequently this is performed through a plasmid intermediate in order to easily produce and purify large amounts of the defective vector genome (Spaete and Frenkel, 1982). To create a packaged defective viral vector, the plasmid is first introduced into cells in tissue culture by transfection. Viral proteins requisite for replication and packaging, as well as structural proteins for the resulting particle, are then provided *in trans* by one or more viral genomes (Spaete and Frenkel, 1982; Kwong and Frenkel, 1995). This can be done either by co-transfection of DNA encoding these proteins with the vector plasmid, or by 'superinfecting' transfected cells with an infectious helper virus (Spaete and Frenkel, 1982; Kaplitt et al., 1991; Kwong and Frenkel, 1995). The resulting viral stock will then contain progeny helper virus mixed with packaged defective viral vectors which contain no viral genes and cannot autonomously replicate. Since the contaminating helper virus represents a continued source of potential toxicity, strategies discussed below have centered upon either using helper viruses of reduced virulence or completely eliminating the helper virus in order to generate a stock of pure defective viral vectors.

3. Defective HSV vectors

The first application of defective viral vectors in neurobiology utilized the defective HSV vector. The HSV genome consists of 150 kilobases (kb) of double-stranded DNA which encodes approximately 75 genes (Roizman, 1990; Roizman and Sears, 1990). As indicated above, the defective HSV vector was originally designed as a result of research into HSV defective interfering particles. When isolated in nature, these particles consisted of multiple copies of a subset of the entire HSV genome packaged into an HSV coat (Spaete and Frenkel, 1982, Spaete and

Fig. 1. The HSV amplicon. The HSV amplicon is a plasmid which contains the HSV origin of DNA replication and the HSV cleavage/packaging signal. No viral gene are contained within the plasmid. In the presence of proteins provided by the HSV helper virus, the amplicon will package into an HSV coat.

Frenkel, 1985; Stow and McMonagle, 1982; Vlazny et al., 1982). Since DIPs required co-infection by an HSV helper virus in order to propagate, it was determined that one or more proteins essential for replication and/or packaging was absent from the fragment of HSV DNA present within the DIP. In order for the fragment to replicate and package, however, it was believed that the requisite cis-acting recognition elements for replication and cleavage/packaging of the DNA into an HSV coat were present in all DIPs (Spaete and Frenkel, 1982, Spaete and Frenkel, 1985; Stow and McMonagle, 1982). After examining the DNA of various DIPs, a common fragment was identified. Insertion of this fragment into a plasmid resulted in replication and packaging of the plasmid DNA into an HSV coat when the plasmid DNA was co-transfected with wild-type HSV DNA in tissue culture (Spaete and Frenkel, 1982, Spaete and Frenkel, 1985; Stow and McMonagle, 1982). Since the entire plasmid was packaged, including any foreign DNA

sequences, and the resulting viral particle could transmit the packaged DNA to a new cell, it was recognized that this system represented an attractive new DNA gene transfer vehicle. Further research eventually demonstrated that this fragment contained an HSV origin of DNA replication (termed the HSV ori) (Stow and McMonagle, 1982, Stow and McMonagle, 1983; Stow, 1985; Elias and Lehman, 1988; Challberg, 1991; Olivio et al., 1991) and a signal for cleavage and packaging of viral DNA (termed the HSV a sequence since it resides within the repeated a subunit of the HSV genome) (Vlazny and Frenkel, 1981; Vlazny et al., 1982; Spaete and Frenkel, 1985; Deiss et al., 1986; Deiss and Frenkel, 1986).

The plasmid upon which the defective HSV vector is based was called an amplicon (Fig. 1). In the presence of proteins provided by the HSV helper virus, the amplicon will package into an HSV coat (Fig. 2). Since approximately 150 kb of DNA must be packaged into an HSV particle, then substantially smaller subunits of DNA containing the requisite ori and cleavage/packaging sequences will be replicated in tandem repeats prior to packaging (Kwong and Frenkel, 1995). Thus if a 10 kb amplicon is used as the genome, roughly 15 copies of this plasmid will be packaged into a single HSV particle. This will result in delivery of 15 copies of the foreign gene of interest to a cell for each individual defective HSV vector particle, thereby 'amplifying' gene delivery. Since the amplicon also contains HSV cis-acting elements without any HSV genes, only the gene product of interest will be expressed in the absence of any HSV gene expression. This became an attractive gene transfer vehicle, particularly for the CNS, since much of the basis for pathogenicity due to direct cytotoxic effects of immune reactions to HSV gene products would be limited.

While these features of the defective HSV vector are attractive, the inability to completely eliminate the helper

Fig. 2. Generation of the defective HSV Vector. The amplicon is first transfected into cells. HSV genome is introduced either by co-transfection or 'superinfection' the following day. Only the helper virus will express the viral genes which will allow replication and packaging of both the amplicon and the HSV genome. The resulting stock consists of both defective vectors and progeny helper virus. A deletion or temperature-sensitive mutant helper virus is usually employed in order to limit toxicity. Since the two particles are virtually identical in size and surface proteins, it is not possible to physically separate the two particles.

virus to date has severely limited the potential advantages of this system. In order to generate the packaged vector, HSV proteins must be provided in trans by either co-transfection of HSV DNA or 'superinfection' with HSV after transfection of the amplicon into cultured cells (Kwong and Frenkel, 1995). The resulting stock then consists of a mix of defective HSV vectors and progeny helper HSV. Initially a stock may contain mostly helper virus, but the amplicon is capable of interfering with helper virus replication. Thus, with subsequent passaging, the ratio of defective to helper virus changes, and by 4–8 passages a stock consisting of greater than 90% defective vectors can be generated (Kwong and Frenkel, 1995). This serves to substantially limit potential problems due to residual helper virus, however clearly toxicity from the helper virus is not entirely eliminated in this system. Physical separation of the two particles is impossible, since the only difference between these two species is the base composition of the DNA. Initially wild-type HSV was used as a helper virus, but even a small number of residual particles can rapidly replicate and spread following CNS injection, resulting in substantial toxicity and death. In order to use the defective HSV vector as a CNS gene transfer vehicle, mutant helper viruses were used (Geller and Breakefield, 1988; Geller et al., 1990; Kaplitt et al., 1991). These mutants allow generation of defective vectors in tissue culture under permissive conditions, however the mutations render the helper viruses inactive in vivo (DeLuca et al., 1985; Paterson and Everett, 1990).

The defective HSV vector has been used for a wide-range of in vivo gene transfer studies. After the initial demonstration that the vector could result in successful gene transfer to non-dividing neurons and glial cells in vitro and in vivo (Geller and Breakefield, 1988; Kaplitt et al., 1991), a variety of functional studies demonstrated the flexibility of this system. Expression of the NGF receptor has resulted in functional recovery of peripheral nerves following axotomy (Federoff et al., 1992; Xu et al., 1994), while expression of both bcl-2 and the glucose transporter have both provided protection from possible ischemic insults in the brain (Ho et al., 1993; Federoff, 1995). Transfer of the gene for tyrosine hydroxylase into the striatum of animals with unilateral lesions of the substantia nigra has resulted in significant behavioral improvement (Geller et al., 1995). The defective HSV vector has also been used as an alternative to transgenic mice for in vivo promoter analysis, with important regions of the rat preproenkephalin promoter identified through in vivo deletion analysis (Kaplitt et al., 1994b; Yin et al., 1995). Finally, insertion of two transcription units into a single vector has resulted in synthesis of two foreign gene products within a single cell (Verhaagen et al., 1995). These represent only a few examples of the numerous successes that have been achieved using the defective HSV vector. The inability to eliminate contaminating helper virus, with the resulting expression of HSV proteins from even mutant helper

viruses in target tissues, led to the search for other defective viral vectors which may permit complete elimination of helper virus.

4. AAV vectors

Adeno-associated virus (AAV) is a naturally occurring defective virus. AAV is a 4.7 kb single-stranded DNA virus (Strivastava et al., 1983) which is usually found in tissues which are also infected by adenovirus, which is the derivation for the name of the virus. Productive AAV infection requires coinfection by adenovirus (Atchison et al., 1965; Melnick et al., 1965; Hoggan et al., 1966) or HSV (Buller et al., 1981; McPherson et al., 1985), which may serve as helper viruses for both wild-type AAV and for the vector described below. Although the precise role of all helper functions has not been fully determined, adenovirus proteins have been shown to be important for transactivating AAV promoters (Richardson and Westphal, 1981; Richardson and Westphal, 1984; Tratschin et al., 1985; Chang et al., 1989; Flint and Shenk, 1989), stabilizing AAV mRNA and enhancing the efficiency of translation of some AAV mRNAs (Strauss et al., 1976; Jay et al., 1979; Myers et al., 1980; Richardson and Westphal, 1981; Jay et al., 1981; McPherson et al., 1982; West et al., 1987; Janik et al., 1989). In the absence of helper virus, the wild-type virus will enter into a latent state from which the AAV genome may later be 'rescued' (Hoggan et al., 1972; Berns et al., 1975; Handa et al., 1977; Cheung et al., 1980). Wild-type AAV can also integrate into a specific site in human chromosome 19 when entering the latent state (Kotin et al., 1990; Samulski et al., 1991), which is unique among DNA viruses. Unlike HSV defective interfering particles, however, AAV is a distinct virus which is much smaller than the helper adenovirus (35 kb) or HSV (150 kb) and which possesses a unique capsid consisting of AAV-specific structural proteins encoded by the AAV genome (Johnson et al., 1971; Johnson et al., 1975; Rose et al., 1971; Tsao et al., 1991).

AAV was an attractive candidate as a vector for gene therapy since wild-type AAV is already defective and has not been associated with any human disease (Berns et al., 1982). Since the AAV chromosome is small (less than 5 kb), the entire genome can easily be cloned into a plasmid. As a DNA virus, AAV possesses cis-acting signals for replication and packaging, however these reside within the 125 bp terminal repeats flanking the rest of the AAV genome (Samulski et al., 1987; Samulski et al., 1989; McLaughlin et al., 1988). When flanked by the AAV terminal repeats, foreign DNA could be packaged into an AAV coat when transfected cells were infected with wild-type AAV and helper adenovirus (Hermonat and Muzyczka, 1984; Tratschin et al., 1985; Samulski et al., 1987; Samulski et al., 1989; McLaughlin et al., 1988). This provided the basis for the AAV vector. As with the

Fig. 3. The AAV Vector. The AAV vector plasmid consists of two AAV terminal repeats which contain the necessary origin of DNA replication and packaging signals. No AAV genes are present within the plasmid. In the presence of AAV replication and structural proteins, the sequences between the terminal repeats will be packaged into an AAV coat.

defective HSV vector, however, residual contamination by wild-type AAV and helper adenovirus represented a continued difficulty. Thus a novel system was developed for creation of a DNA viral vector which is completely free of contaminating helper virus.

The AAV vector as currently practiced is based upon a two plasmid system (Samulski et al., 1989). The vector, as described above, contains foreign sequences of interest up to 5 kb flanked by the 125 bp AAV terminal repeats (Fig. 3). When oriented properly the DNA between the repeats will be packaged into an AAV coat. A second plasmid is then used called the 'helper plasmid'. This consists of the

entire AAV genome without the terminal repeats (Fig. 4). The helper plasmid can thus express all of the AAV proteins necessary for replication, packaging and creation of new particles, however the absence of terminal repeats prevents the helper plasmid from packaging. Co-transfection of these two plasmids will thus result in replication and packaging of the vector into an AAV particle, while the AAV genes in the helper plasmid will not be packaged and will thus be lost (Fig. 4). Finally, infection by adenovirus is still necessary for this process to efficiently occur (Samulski et al., 1989). Thus the resulting vector stock consists of defective AAV vectors with no AAV genes, and progeny helper adenovirus (Fig. 4). Unlike the defective HSV vector, however, the contaminating helper virus in this instance is completely distinct from the vector (Samulski et al., 1987; Samulski et al., 1989). For example, the stock is first heated to 56°C for 30–45 min, which will inactivate the adenovirus while leaving the AAV intact. Then cesium chloride centrifugation permits physical separation of the inactivated adenovirus from the AAV vector due to the vastly different particle sizes. Following dialysis to remove the cesium chloride, a stock of helper-free defective AAV vectors is obtained (Bartlett and Samulski, 1995). Thus AAV represents the first DNA viral vector which contains no viral genes and is not contaminated by helper virus, thereby eliminating pathogenicity due to toxicity or immunogenicity of viral gene products.

The AAV vector has only recently been applied to neurobiology. While relatively new to the field of gene

Fig. 4. Generation of AAV Vectors. The AAV vector is transfected into cells. At the same time a second plasmid is transfected, called the helper plasmid. The helper plasmid contain all of the AAV coding regions but lacks terminal repeats. Thus the helper plasmid can support AAV replication and packaging but cannot itself replicate and package. Finally, adenovirus infection is required for efficient expression of the AAV genes from the helper plasmid. The resulting stock consists of the fully defective AAV vector and progeny helper virus, but no wild-type AAV is present. The adenovirus can then be removed from the stock resulting in a pure population of defective AAV vectors.

therapy in general, AAV vectors have been used for several applications outside of the CNS. For example, AAV vectors have been shown to efficiently transduce bone marrow progenitor cells for the purpose of either correcting hematologic disorders with a genetic basis or to provide proteins to the body via blood cells (Zhou et al., 1993; Goodman et al., 1994; Miller et al., 1994). Since AAV can infect airway epithelium in a manner analogous to adenovirus, AAV vectors have generated interest as agents for transfer of the CFTR gene in order to treat cystic fibrosis (Flott et al., 1993). More recently, AAV vectors have been used for gene transfer into the CNS in vivo (During et al., 1994a; Kaplitt et al., 1994a). While some transduction of glial cells was demonstrated, the vast majority of modified cells appeared to be neuronal (Kaplitt et al., 1994a). Further examination of this phenomenon has suggested that there may be regional influences upon both the efficiency and cell-specificity of AAV-mediated transduction in vivo. Finally, long-term therapeutic improvement in the rat 6-hydroxydopamine model of Parkinsonism following tyrosine hydroxylase gene transfer into the striatum suggests that AAV vectors may be useful as potential agents for safe and efficient gene therapy of CNS disorders (Kaplitt et al., 1994a).

5. Future directions

This review has been intended to outline the current state of the art with respect to defective viral vectors as gene transfer agents, particularly for study and possible treatment of the central nervous system. Certain applications have also been presented, although an exhaustive review of all applications to date was not intended. Gene therapy is a rapidly evolving field, however, and the number of researchers using these techniques as well as the variety of applications is exponentially expanding. Currently no single gene transfer vehicle is ideal, and numerous laboratories are investigating issues common to all gene transfer strategies such as creating synthetic, cell-specific promoters or modification of viral surface proteins. Since defective viral vectors represent important agents for safe and efficient gene transfer, however, rapid advances in the current technology will doubtless be achieved. For example, promising data suggesting the possibility of a helper-free defective HSV vector has already been generated. Furthermore, since the requisite cis-acting elements for most DNA viruses have been identified, it will be relatively simple to generate defective vectors based upon other DNA viruses. Many of these systems will be quite inefficient at either creating vectors or transferring genes to target cells. Thus future work will concentrate upon applying the appropriate vector to the question at hand and then maximizing the efficiency of that system for the given purpose. As is evident from this review, however, the desire for optimized systems should not deter

those interested in using defective viral vectors at present, since the current state of the art permits numerous novel approaches to the study of the central nervous system.

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