[23] Adeno-Associated Virus Vectors for Short Hairpin RNA Expression

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Abstract

Five recent publications have documented the successful development and use of gene transfer vectors based on adeno-associated virus (AAV) for expressing short hairpin RNA (shRNA). In cultured mammalian cells and in whole animals, infection with these vectors was shown to result in specific, efficient, and stable knockdown of various targeted endo- or exogenous genes. Here we review this exciting approach, to trigger RNA interference *in vitro* and *in vivo* by shRNA expressed from AAV vectors, and describe the state-of-the-art technology for vector particle generation. In particular, we present a set of novel AAV vector plasmids that were specifically designed for the easy and rapid cloning of shRNA expression cassettes into AAV. The plasmids contain alternative RNA polymerase III promoters (U6, H1, or 7SK) together with a respective terminator sequence, as well as stuffer DNA to guarantee an optimal vector size for efficient packaging into AAV capsids. To provide maximum versatility and user-friendliness, the constructs were also engineered to contain a set of unique restriction enzyme recognition sites, allowing the simple and straightforward replacement of the shRNA cassette or other vector components with customized sequences. Our novel vector plasmids complement existing AAV vector technology and should help further establish AAV as a most promising alternative to using adeno- or retro-/lentiviral vectors as shRNA delivery vehicles.

Introduction: Exploiting RNA Interference (RNAi)—Promises and Problems

Over the past 3 years, the field of nucleic acid-based inhibitors of gene expression has seen an unprecedented wave of interest, sparked by a groundbreaking study by Elbashir *et al*. (2001). First, their report provided initial proof that mammalian cells are capable of eliciting the sequence-specific posttranscriptional gene silencing pathway known as RNA interference (RNAi), which in the past had been assumed to be restricted to lower organisms and plants. Second, they also discovered that the RNAi pathway becomes efficiently activated by the introduction of short double-stranded RNAs (dsRNAs), which mimic products of the Dicer endonuclease and as

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such trigger the targeted degradation of complementary mRNAs that is characteristic of RNAi.

It was perhaps this particular aspect of their work that has fueled the most interest in RNAi, because it paved the way for the subsequent straightforward exploitation of this natural mechanism as a powerful novel research tool. A most important successive finding was that besides in the form of small dsRNAs, two complementary RNA strands are also effective triggers of RNAi when present as a single stem-loop [short hairpin RNA (shRNA); Paddison *et al*., 2001]. As such, they can be easily generated intracellularly by expression from RNA polymerase II or III promoters such as CMV or U6. This overcomes the main drawbacks associated with synthetic dsRNAs, namely, the transient nature of gene silencing and high costs for dsRNA manufacturing. Moreover, it offers wide possibilities to control the on- and offset of RNAi and to restrict the effect to particular tissues, although so far there is only limiting evidence that these goals will soon be met.

Significant advances were subsequently also made in the design of the shRNA molecules themselves, particularly with regard to the issues of functionality and specificity. Thus, the large-scale systemic analysis of shRNAs targeting defined mRNAs recently led to the identification of characteristics associated with high effector molecule functionality, culminating in the establishment of novel algorithms for the rational design of potent shRNAs (e.g., Ding *et al*., 2004; Reynolds *et al*., 2004). In parallel, others have developed novel enzyme-mediated strategies for generating numerous functional shRNA constructs from any gene of interest, which bears the inherent advantage that no prior knowledge about target transcripts is needed (Luo *et al*., 2004; Sen *et al*., 2004; Shirane *et al*., 2004). However, identification of the best effector molecules from an shRNA library will not be easy and straightforward.

Irrespective of the exact shRNA selection method, a critical problem becoming increasingly apparent is that of potential specific or unspecific adverse effects from expression of shRNAs in cells or whole animals. Reviewing this important topic in more detail will be beyond the scope of this chapter, but note that recently observed side effects include activation of arms of the interferon or the microRNA pathways, as well as unwanted off-targeting of endogenous cellular genes with partial or full homology to the actual target (e.g., Doench *et al*., 2003; Jackson *et al*., 2003).

Despite this crucial concern, the previously mentioned advances and refinements in design of shRNA expression cassettes have dramatically accelerated the pace at which this novel technology is being applied as an analytical and a therapeutic tool. The enormous promise of the overall approach has already been documented by a recent wealth of reports. For

instance, various groups have addressed the great potential of RNAi as a revolutionary method for studying gene functions in whole animals, in which its advantage over conventional knockout models is that more target genes can be assessed in less time, thus saving efforts and resources. Most outstanding publications reported the generation of shRNA libraries targeting thousands of human or mouse genes at one time (Berns *et al*., 2004; Paddison *et al*., 2004), providing hope that the ultimate challenge can be met—to perform RNAi-based large-scale loss-of-function genetic screens of the entire human genome.

The feasibility to effectively and specifically silence endo- or exogenous genes also holds great promise for treating viral infections, cancers, and inherited genetic disorders, leading to the anticipation that RNAi will emerge as a novel powerful tool for gene therapy. The potential of this approach is exemplified and underscored by studies demonstrating the successful use of shRNA to inhibit replication of, or gene expression from, human pathogens such as HIV or hepatitis B virus (Lee and Rossi, 2004; McCaffrey *et al*., 2003). Proof of principle has likewise already been provided that RNAi can be exploited to knock down cellular or viral oncogenes (reviewed by Friedrich *et al*., 2004) or to repress dominant gain-of-function gene mutants causing human disorders (Miller *et al*., 2003).

Although these examples manifest the tremendous promise of RNAi for various applications, the key challenge that needs to be met before the approach can become widely accepted is to develop systems that will efficiently deliver shRNA expression cassettes to mammalian or, ultimately, human cells. To overcome this hurdle and put such a delivery tool in place is central to establishing RNAi-based gene therapies, as exemplified by scenarios in which shRNAs will be used to knock down oncogenes expressed within tumor cells or viral genes within infected cells. Obviously, the success of these strategies will largely depend on the ability to hit each target cell, and this goal becomes even more challenging when the approach will be translated to whole organisms, thus requiring tools to effectively deliver and express shRNAs *in vivo*.

However, this goal can unlikely be met with the currently available technologies for expression of shRNAs from plasmid DNAs or from any other form of nonviral vector. Although these constructs are easy to generate and useful for studies in isolated cultured cells, there is no methodology on hand to efficiently deliver nonviral vectors to the entire cell mass within an intact organ *in vivo*. Consequently, more researchers have recently begun to turn their attention to developing virus-derived shRNA expression vectors, based on the promise that as compared with their nonviral counterparts they will provide a significantly higher efficiency for shRNA delivery in whole organisms. In addition, it is hoped for that

using a recombinant virus as delivery system will permit better control over tissue specificity of shRNA expression, because most viral vectors currently under development are either naturally characterized by distinct tissue tropisms or can relatively easily be genetically modified and retargeted to particular cell types (e.g., Nicklin and Baker, 2002).

The viral vectors recently engineered as shRNA delivery vehicles comprise three major classes of viruses: adenovirus, retro- or lentivirus, and adeno-associated virus (AAV). The first two viral vector types have already been studied rather extensively as tools for RNAi-mediated gene silencing and are the subject of chapters 9 and 13. Here, we focus on the AAV vector system. We firstly briefly review wild-type and recombinant AAV (rAAV) biology, then discuss and describe the use of AAV vectors as mediators of shRNA expression, and finally give detailed protocols to generate such vectors.

Adeno-Associated Virus (AAV)—From Wild-Type Virus to Recombinant Vectors

Wild-type AAV and vectors derived thereof have already been extensively reviewed over the past 20 years (e.g., Berns and Linden, 1995; Grimm and Kleinschmidt, 1999), and we will limit the information provided here to a minimum. Unless otherwise specified, all facts and numbers presented refer to AAV serotype 2 (AAV-2), which is considered the prototype of the AAV group. Figure 1 provides a comprehensive summary of the main characteristics of wild-type and recombinant AAV (rAAV).

AAVs belong to the family Parvoviridae, which encompasses viruses with nonenveloped, icosahedral capsids with a diameter of about 20 nm, containing a linear single-stranded DNA molecule. In line with the small capsid size, the viral genome is particularly short (only 4681 nt long) and is also relatively simple in its organization, comprising only two genes (*rep* and *cap*) that encode four nonstructural (Rep) and three structural (VP) proteins. The two genes are embedded between two inverted terminal repeats (ITRs) 145 nt long, which typically assume a T-shaped hairpin secondary structure. ITRs play crucial roles in many steps of the viral life cycle, beginning with the expression and replication of the viral genome early in a productive infection and followed by its packaging into the AAV capsid. In subsequently infected cells, they are involved in the conversion of the single-stranded viral DNA into a double-stranded form from which AAV gene expression occurs, and which eventually becomes integrated into the host chromosome or persists episomally.

Besides its small size and simple organization, AAV is further distinguished from other human viruses by its requirement for a helper virus to

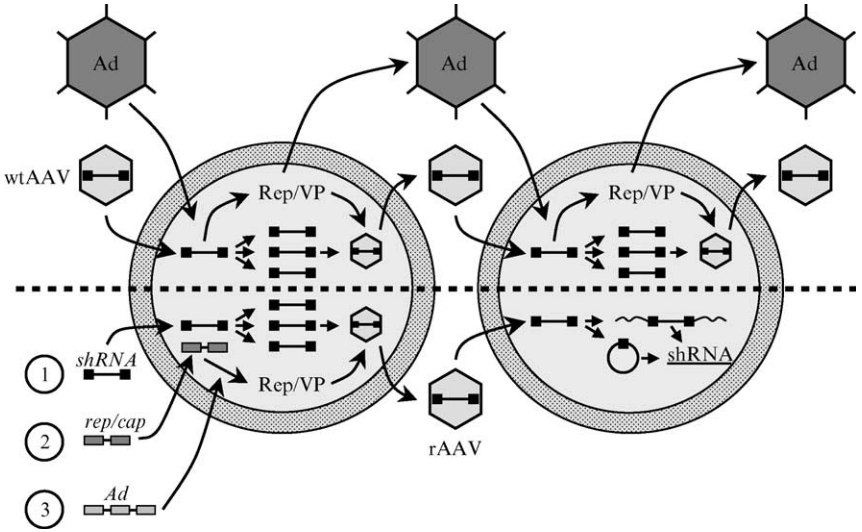

FIG. 1. Life cycles of wild-type (wtAAV) and recombinant adeno-associated virus (rAAV). The two pairs of circles represent mammalian or human cells; the nuclei are depicted by the lighter inner circles. (*Top*) Wild-type AAV. To propagate wild-type AAV, cells are coinfected with the virus and a helper virus (here adenovirus). The AAV capsid trafficks (not shown) to the nucleus, where it releases its genome [black line with boxes at the end, representing the two inverted terminal repeats (ITRs)]. Products expressed from the helper virus support expression of the AAV genes, resulting in large amounts of AAV replication (Rep) and capsid (VP) proteins. In parallel, the wild-type AAV genome replicates to high copy numbers in the nucleus and eventually becomes encapsidated in virions formed by assembled VP proteins. The helper virus finally induces cell lysis, and progeny AAV as well as replicated adenovirus are released to start a fresh infection cycle in another cell. (*Bottom*) Recombinant AAV. The generation of rAAV requires the same components that are needed to propagate wild-type virus. However, for rAAV production, they are provided from three separate plasmids, which are triple transfected into cells: (1) an AAV vector plasmid comprising the transgene (here labeled as shRNA) flanked by the AAV-2 ITRs, (2) an AAV helper plasmid encoding the *rep* and *cap* genes, and (3) an adenoviral helper plasmid (typically encoding the E2A, VA, and E4 genes). In the cell nucleus, the AAV helper expresses Rep and VP proteins *in trans* (supported by adenoviral helper functions), whereas the ITRs replicate the transgene (shRNA) sequences *in cis*. Similar to the wild-type scenario, the VP proteins then assemble into capsids, which take up the amplified viral DNA. In contrast, the cells do not produce progeny helper virus and thus do not get lysed, allowing the rAAV particles to be harvested from the intact cell (not shown). In a reinfected cell, the rAAV particle releases its genome, which subsequently does not get replicated (because of the absence of AAV or adenoviral gene expression), but instead can integrate into the host chromosome or assume extrachromosomal circular or linear (not shown) forms, either of which results in rAAV genome persistence.

complete its own life cycle in the coinfected cell. The helper virus, typically adenovirus or a member of the herpes virus family, provides products (RNAs and proteins) that stimulate gene expression from the AAV promoters, enhance transport and splicing of the AAV pre-mRNAs, and in case of herpes simplex virus 1 support replication of the AAV genome (Weindler and Heilbronn, 1991). AAV's dependence on a pathogenic helper virus is particularly unique, considering that no AAV has ever been associated with any human malignancy, although AAV serotype 2 is believed to be an extremely common virus. Previous studies have estimated that up to 80% of the human population is seropositive for this particular AAV serotype (Erles *et al.*, 1999).

This detail may have led to the very early definition of AAV-2 as the prototype of this virus class, despite the fact that along with its discovery in the late 1960s, other serotypes had been isolated as well (AAV-1 to -4). The AAV group has further grown with the description of AAV-5 to -8 between 1984 and 2002 (reviewed by Grimm and Kay, 2003). The most significant expansion came with a report describing the isolation and identification of 108 new AAV variants, of which 55 were found in human tissue and 53 in nonhuman primates (Gao *et al.*, 2004). This distribution is in line with the hypothesis that AAV-1, -4, -7, and -8 are simian viruses, whereas the other four previously described AAV serotypes seem to primarily infect humans.

The early discovery that AAV ITRs are the only sequences required in *cis* to mediate packaging of DNA sequences embedded in between has paved the way for generating vectors derived from wild-type AAV. Thus, a straightforward approach to produce a rAAV vector genome is to replace the entire wild-type *rep* and *cap* genes with any sequence of choice. To facilitate this step, a large number of AAV vector plasmids have been generated over the years that contain the AAV-2 ITRs as the sole sequences derived from the wild type, and, in addition, carry unique restriction enzyme recognition sites, allowing the easy insertion of foreign DNA between the ITRs (e.g., Zolotukhin *et al.*, 1996). The only important consideration to be made at this step is that the total size of the resulting recombinant cassette, including the transgene and the two ITRs, must not significantly exceed the size of the AAV wild-type genome, to guarantee its efficient encapsidation. This is a direct consequence of the fact that the AAV capsid is an icosahedral structure and can only accommodate a particular amount of DNA; accordingly, the packaging limit for AAV vectors is approximately 4.8 kb of foreign DNA. As discussed later, this has important implications for the design and generation of AAV vectors for the expression of shRNA.

To package the recombinant genome into viral capsids, the AAV vector plasmid is typically transfected into cultured cells together with two other

plasmids: an AAV helper plasmid encoding the *rep* and *cap* genes in *trans* and an adenoviral plasmid providing the respective helper functions. During the subsequent incubation of transfected cells, the Rep and VP proteins expressed from the AAV helper plasmid replicate the recombinant genome to high copy numbers and form AAV capsids for its packaging, respectively. The products expressed from the adenoviral plasmid basically provide the same helper functions as do infectious adenovirus during growth of wild-type AAV; that is, they enhance expression of the *rep* and *cap* genes as well as support AAV RNA transport and splicing. However, the adenoviral plasmid is no longer infectious, thus ensuring that the final rAAV preparation is free from contaminating adenovirus. Likewise, the rAAV stocks are also free from wild-type AAV virus, because the *rep/cap*-expressing helper plasmid lacks the ITRs required for DNA encapsidation (see also Fig. 1).

The approach to generate AAV vector particles by transient transfection of cultured cells has become a standard methodology and is most widely used, yet manifold variations of this concept have been tried and proposed throughout the years. A detailed description of these alternative protocols is beyond the scope of this chapter, but two most important improvements should be mentioned. First, advances were made regarding the number of plasmids required to generate the AAV vector particles. For instance, work from our group has shown that it is feasible to express the AAV and adenoviral helper functions from a single-hybrid plasmid, which reduces the number of constructs to be transfected to two (Grimm *et al.*, 2003a). It is even possible to incorporate the AAV vector sequences into the same plasmid and thus produce rAAV particles from a single construct, thus significantly saving time and costs (Grimm and Kay, unpublished).

Second, it was found that the transfection-based protocol for rAAV generation can easily be adapted to produce AAV vector particles that are derived from naturally occurring AAV serotypes other than AAV-2 (Grimm and Kay, 2003). The only component of the previously outlined protocol that needs to be modified in order to produce an AAV vector based on an alternative serotype is the capsid gene within the AAV helper plasmid. In contrast, the *rep* gene in the helper and the ITRs in the vector plasmid remain unchanged. The resulting recombinant particle thus consists of an AAV-2-based vector DNA packaged into a capsid from another serotype and is referred to as an AAV pseudotype; the production process is called cross-packaging (Grimm, 2002). The benefit of this approach is that the resulting rAAV particles display unique tropisms and serological properties that are different from those of AAV-2. This largely expands the range of potential targets for AAV-mediated gene transfer and will help circumvent immunological issues, which should both be important with respect to the use of AAV vectors to deliver shRNAs.

AAV Vectors for shRNA Expression *In Vitro* and *In Vivo*

In this section, we discuss the specific properties of rAAV that make this vector system particularly promising for shRNA expression and then review the recent literature demonstrating successful use of AAV-based vectors for shRNA transfer *in vitro* and *in vivo*.

Theoretical Considerations

In theory, an ideal vector system for delivery of shRNA expression cassettes should fulfil four requirements. It should be (1) efficient, (2) safe, (3) allow stable shRNA expression, and (4) the vector itself should be easy to manufacture. From our experience with rAAV-mediated transgene delivery gained in previous studies, there are reasons to believe that the AAV vector system might meet all these goals.

First, a wealth of data supports the notion that AAV vectors are extremely efficient tools for gene delivery *in vitro* and *in vivo*, as defined by the variety of cell types that can be transduced as well as by the number of recombinant vector genomes that can be introduced into, and maintained in, each target cell.

In particular, AAV serotype 2, which has till now served as the basis for most vectors derived from AAV, has a very broad host range and is able to infect both dividing and nondividing cells, including important therapeutic targets such as hepatocytes or neurons (e.g., Grimm *et al*., 2003b). rAAV has the specific ability to transduce quiescent cells, and its use for gene transfer provides a clear advantage over using retroviral vectors, which require cell division for efficient transduction.

Moreover, the feasibility to easily cross-package a given AAV vector construct into capsids from other alternative AAV serotypes further expands the host range of this vector system, because the known AAV serotypes are functionally and serologically distinct from each other. For instance, AAV serotype 1 is most efficient for muscle-directed gene transfer and thus is the choice for pseudotyping when targeting this tissue, whereas AAV-8 appears superior among all serotypes for liver transduction (Gao *et al*., 2002). In addition, beyond using naturally occurring AAV variants, multiple groups have succeeded in genetically altering the AAV-2 capsid through insertion of customized ligand sequences. This resulted in ablation of the natural AAV-2 tropism while allowing the transduction of cell types that had previously been refractory to infection by AAV-2 (e.g., Perabo *et al*., 2003). The results from all these approaches together make it tempting to speculate that theoretically any given target cell type can be transduced by, and made to express shRNAs from, an AAV vector.

In addition, previous reports also demonstrated that transduction with AAV vectors can result in high copy numbers of the recombinant transgene in the infected cell. For example, the use of AAV-8 to transduce hepatocytes *in vivo* has been reported to lead to individual cells carrying more than 10 vector copies, which were stably maintained over time (Gao *et al.*, 2002). With respect to expression of shRNA, it is currently unclear as to what an optimal number of vector copies per cell will be, as this will certainly depend on specific parameters such as strength of promoter used to drive the shRNA. Nevertheless, it is reasonable to assume that the AAV vector system has the potential to deliver and maintain sufficiently high shRNA copy numbers in the transduced cell.

Last but not least, a high efficiency of the AAV vector system is also provided by the fact that the use of different serotypes or capsid variants permits the readministration of recombinant particles (Grimm and Kay, 2003). This is crucial considering that administration of a particular AAV vector type will result in a humoral immune response, characterized by formation of antibodies against this specific serotype, which will prevent repeated vector delivery. However, switching to a capsid from another serotype will allow to circumvent this immune response, thus enabling readministration of a given AAV vector genome to the same organism. In regards to RNAi, this might provide a useful strategy to deliver multiple different shRNAs to the same host in a timely coordinated manner or it will allow subsequent administration of shRNA cassettes and regulatory elements, such as transcriptional activators or repressors. Theoretically, it will also allow expression of shRNAs from AAV in patients having preexisting immunity against a particular AAV serotype. This is an important option when considering the previously mentioned highly prevalent seropositivity against AAV-2 in the human population, which may pose a major hurdle to the widespread therapeutic application of AAV-2 vectors.

Second, rAAVs are regarded to be among the safest of all known viral-based vector systems. This is partly because the wild-type virus has never been associated with any human malignancy, in contrast to all other viruses that are currently being exploited as vectors, such as adenovirus or lentivirus. A more direct proof of the high safety profile of wild-type and rAAV was recently provided by Stilwell and Samulski (2004), who used DNA microarrays to identify and directly compare genes modulated during AAV or adenovirus infection of cultured cells. Impressively, AAV infection elicited a nonpathogenic response whereas adenovirus infection resulted in induction of immune and stress-response genes associated with pathogenesis. In this respect, it should be clarified that although adenoviral helper functions are needed during rAAV production, the resulting AAV

vector stocks are free from any contaminating virus because of the inability of the adenoviral plasmid to replicate.

Moreover, the AAV system is also distinguished from the other viruses under development in that vectors derived from wild-type AAV only retain the ITRs but do not express any viral genes and thus are gutless by design and definition. Because of the lack of viral gene expression, AAV vectors do not cause a cytotoxic cellular immune response in the transduced host. The importance of this property can be most dramatically exemplified by recent adverse effects observed in a patient treated with a second-generation adenovirus that was expressing viral genes, although it has not been fully resolved whether the genetic cargo or the viral capsid itself was responsible for the patient's death (Marshall, 1999).

Furthermore, AAV vectors also appear to display only a very modest frequency of integration into the host genome, which further contributes to the overall safety of this particular vector system. The exact fate of the recombinant genome in the transduced cell will, however, depend on many parameters such as cell type and particle dose. For instance, our group has previously shown that in the transduced liver, only a very small fraction (<10%) of all persisting AAV genomes integrate into the hepatoctye genome, whereas the majority assumes extrachromosomal circular DNA forms (Nakai *et al*., 2001). The important topic of viral vector integration is currently being widely studied, partly because of the outcome of another recent clinical study. In this case, treatment of patients with a retroviral vector led to integration of the viral genome into the host chromosome, followed by the adverse activation of a protooncogene (Kaiser, 2003). This underscores the importance of using a vector for long-term shRNA transfer that displays only low frequencies of random integration, such as rAAV.

Third, the property of AAV to persist in the infected cell as extrachromosomal forms usually results in stable and strong expression of the encoded transgene. Numerous reports have demonstrated maintained expression of AAV-delivered transgenes for over 18 months in mice (Snyder *et al*., 1999) or dogs (Mount *et al*., 2002). For RNAi applications, the feasibility of achieving persistent shRNA expression from a viral vector will be highly desirable, unless in scenarios in which transient expression is specifically intended. For most applications, however, continued expression of the transduced shRNA will be the goal, as exemplified by settings in which RNAi will be used to inhibit oncogenes or genes expressed from pathogenic viruses.

Fourth, another potential benefit of using the AAV vector system to express shRNAs is the great ease with which recombinant particles can be produced in bulk amounts. Thus, a typical medium-scale rAAV preparation that can be performed in each laboratory will typically result in at least 10^{12}

pure recombinant vector particles. (See ''Generating shRNA-Expressing AAV Vectors: A Protocol'' for details.) It is impossible to exactly predict the amount of AAV vector needed for a specific RNAi application, as this will depend on many parameters, including the choice of an AAV serotype permitting efficient target transduction and the number and accessibility of target cells within the organism. Nevertheless, considering that the transfection method has already been used to manufacture AAV vector for several clinical studies in humans and that upscaling of the protocol to yields of more than 10^{13} total particles is also feasible (Potter *et al.*, 2002), it is reasonable to believe that vector production will not be a limiting factor for the use of AAV for RNAi applications.

Practical Examples

To date, there have been five independent reports on the use of AAV-based vectors for shRNA expression in various target cells *in vitro* and *in vivo* and all highlight the great potential of the approach.

The first study by Tomar *et al.* (2003) was simple in design, but provided the important initial proof of principle that AAV-2 vector particles can be engineered to express shRNA. The authors generated AAV-2 vector plasmids expressing shRNAs directed against p53 or caspase-8, under the control of the H1 or U6 promoter, respectively. Subsequently, they used the particles to infect cultured HeLa cells and demonstrated the expected efficient and dose-dependent knockdown of p53 and caspase 8 proteins by Western blot analyses.

A clinically more relevant study, and also the first report of *in vivo* expression of shRNA from AAV, then came from Hommel *et al.* (2003). This group targeted the dopamine synthesis enzyme tyrosine hydroxylase (Th) by expressing respective shRNAs from AAV-2 vectors in adult mice. Most noteworthy, following stereotaxic injection of the vector into the substantia nigra compacta, Th knockdown was found to be effective and persistent, lasting as long as 50 days. Equally impressive was the finding that the vector-induced reduction of Th protein elicited behavioral defects in the treated mice and created a phenotype reminiscent of rodent models of Parkinson's disease. Thus, this study established the potential of shRNA-expressing AAV vectors to rapidly and easily create spatially restricted gene knockdowns *in vivo* and to test new genetic disease models.

The usefulness of the AAV/shRNA system was further underscored by two independent *in vitro* studies subsequently published by Boden *et al.* (2004) and Pinkenburg *et al.* (2004). The two groups designed AAV-2 vectors to express shRNAs directed against the human immunodeficiency virus type 1 (HIV-1) *tat* gene and the NF-κB p65 subunit, respectively.

Specific and efficient inhibition of HIV-1 replication or interleukin-8 production (as a marker for p65 knockdown) was then demonstrated in cultured primary human lymphocytes and bronchial epithelial cells, respectively.

The latest relevant report by Xia *et al.* (2004) provided the second proof for the efficiency of AAV vectors for expressing shRNA *in vivo*. Similar to the first study by Hommel *et al.* (2003), the vectors were evaluated by using a mouse model of a human dominant neurodegenerative disease, here spinocerebellar ataxia type 1 (SCA1), and vector efficacy was demonstrated by showing reduction in target protein (ataxin-1) expression and by documenting improvements in cellular and behavioral characteristics. An important difference between the two studies was that Xia *et al.* (2004) used a pseudotyping approach to cross-package their shRNA-expressing vector construct into AAV-1 capsids. This enabled them to efficiently target Purkinje cells in the murine cerebellum and to exploit the increased expression kinetics from this particular AAV serotype. Together, this underscores the great promise of pseudotyping shRNA-expressing AAV vectors to achieve targeted and controlled RNAi induction *in vivo*.

Generating Short Hairpin RNA (shRNA)-Expressing AAV Vectors: A Protocol

We describe the materials and protocols needed to generate shRNA-expressing vectors through transient transfection of cultured cells. This represents the most widely used approach for rAAV production and was thus chosen to be reported here.

Materials

Cells. The most commonly used cell type for rAAV production are 293 cells, because they are highly transfectable and contain an integrated copy of the adenoviral E1 gene, which enhances gene expression from the AAV helper plasmid. An alternative is provided by using 293T cells, which differ from 293 cells by an additional copy of the SV40 large T antigen. This decreases the doubling time of 293T cells as compared to 293 and also renders them even more susceptible to calcium phosphate-mediated DNA transfection. For either cell type, it is crucial to maintain the cells at a low passage number (<50) to avoid decreases in the efficiency of rAAV production per cell. It is thus advisable to obtain such a low-passage stock, for example, from a commercial source such as Microbix Biosystems (Toronto, Canada) and then store a working cell bank in aliquots in liquid nitrogen. Moreover, it is important to prevent the cells from growing to confluency, which will otherwise negatively affect their health and the ability to take up DNA.

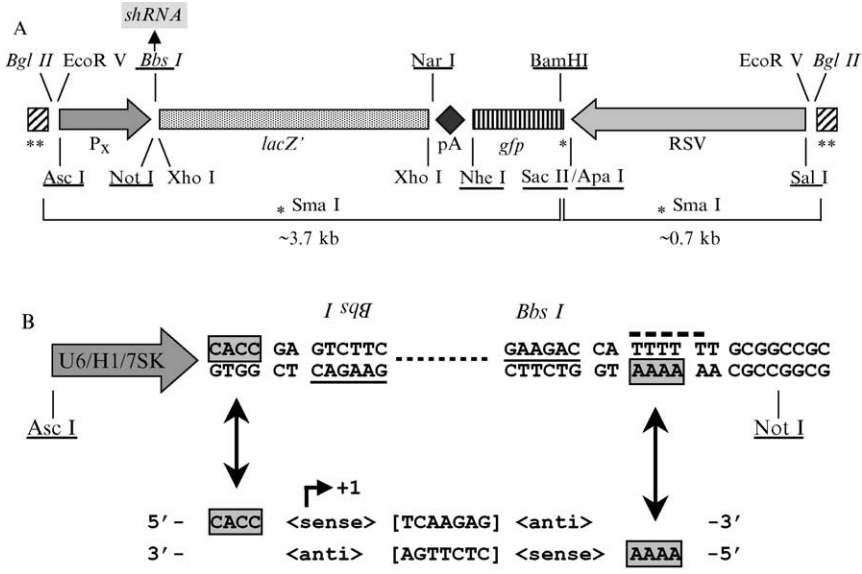

FIG. 2. Novel AAV vector plasmids for short hairpin RNA (shRNA) expression. (A) General vector plasmid scheme. Depicted is our novel AAV vector construct with the AAV-2 ITRs at both ends (hatched boxes), an RSV (Rous sarcoma virus) promoter-driven *gfp* gene (including polyadenylation site; pA), a 2.2-kb stuffer fragment from the *E. coli lacZ* gene, and an RNA polymerase III promoter at the left end (P_x). Three versions of this plasmid were made, carrying the human U6, H1, or 7SK promoter to drive shRNA expression. Also shown are the locations of various restriction enzyme recognition sites within the constructs, with unique sites underlined. The location of the multiple *Sma* I sites is indicated by asterisks, and expected fragments resulting from *Sma* I digestion are shown at the bottom of the scheme. As explained in the text, *Sma* I digestion is useful to confirm ITR integrity after cloning an shRNA insert. Note that one *Sma* I site is located at the start of the *gfp* gene and will be lost on replacement of this gene with customized sequences. The two close *Bbs* I sites (indicated as one) located downstream of the polymerase III promotor serve to insert shRNA sequences in the form of annealed oligonucleotides [see (B)]. All versions of the plasmid express the ampicillin-resistance gene (not shown). (B) Scheme for cloning shRNAs. Shown is a magnified view of the left end of the AAV vector plasmid depicted in (A). As evident, the polymerase III promoter (U6, H1, or 7SK) is followed by two mirrored *Bbs* I sites (the actual recognition sequence is underlined), which are separated by a short spacer region (depicted as a dotted line), and followed by a T_5 terminator sequence (marked by dashed line). Cutting of the plasmid(s) with *Bbs* I leaves two 5′ overhangs, which are highlighted by grey boxes. To clone an shRNA into the *Bbs* I-linearized construct, two complementary oligonucleotides are annealed (see text for protocol), comprising a sense and antisense strand separated by a short hairpin spacer. The oligonucleotides must be designed to display *Bbs* I-compatible overhangs following their annealing, also depicted by grey boxes. Following cloning of the annealed oligonucleotides into the AAV vector plasmid, the shRNA transcription start will be located immediately downstream of the 5′ *Bbs* I overhang (indicated by +1).

In our laboratory, we grow 293 and 293T cells in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with L-glutamine to a final concentration of 2 m*M* and containing fetal bovine serum (10% final volume, HyClone, Logan, UT), penicillin-streptomycin, and antibiotic-antimycotic reagent (Gibco-Invitrogen, Carlsbad, CA). For routine culturing, as well as for rAAV production, the cells are plated in T225 flasks (Corning, Fountain Valley, CA) and passaged when reaching approximately 90% confluency.

Plasmids. The three types of plasmids required for rAAV production have been mentioned earlier [see ''Adeno-Associated Virus (AAV): From Wild-Type Virus to Recombinant Vectors'' and Fig. 1]: (1) an shRNA-encoding AAV vector plasmid; (2) an AAV helper plasmid expressing the capsid gene from the desired AAV serotype, together with the AAV-2 *rep* gene; and (3) an adenoviral helper plasmid. In the next section, we provide guidelines and a protocol to generate the shRNA-expressing vector plasmid. For information on the two helper constructs, we refer the reader to a review article that provides respective background information and, importantly, lists sources for such plasmids (Grimm, 2002).

In our hands, we found it sufficient to purify all three types of plasmids from the bacterial lysate by using purification kits available from Qiagen (Valencia, CA); the use of cesium chloride (CsCl) gradients or special endotoxin-free protocols is superfluous. We typically grow the plasmid-containing bacteria (DH10B, Invitrogen) on a 2.5-L scale and use the Plasmid Giga Kit (Qiagen) for purification, which yields up to 10 mg plasmid DNA. Considering that a vector preparation requires 1.25 mg of each plasmid (see later), this yield is sufficient for up to eight production runs.

Moreover, we routinely check the integrity of the AAV-2 ITRs in our vector plasmids by restriction digest analyses, which is indicated as the sequences are highly prone to recombination and subsequent deletion. A useful enzyme for this purpose is *Sma* I, which cuts twice within one arm of each AAV-2 ITR hairpin. Most often, this particular region is lost by deletion in one of the two ITRs, which is then readily detected by analyzing the *Sma* I digestion pattern (compare Fig. 2A). In contrast, direct sequencing of the AAV-2 ITRs as an alternative method is prevented by the high degree of secondary structure of the sequences, thus making the method unfeasible.

Method

In this section, we provide detailed protocols for the four basic steps required to generate shRNA-expressing AAV vector particles: (1) initial cloning of a recombinant shRNA-encoding AAV plasmid, (2) subsequent packaging of this construct into AAV capsids by transfection of cells,

(3) vector particle purification, and (4) titration. The protocols are very basic and not specific for any particular AAV serotype; the reader is referred to the literature for more information on alternative approaches (Grimm, 2002; Grimm and Kay, 2003). The flowchart in Fig. 3 summarizes the rAAV production steps and shows an approximate time frame.

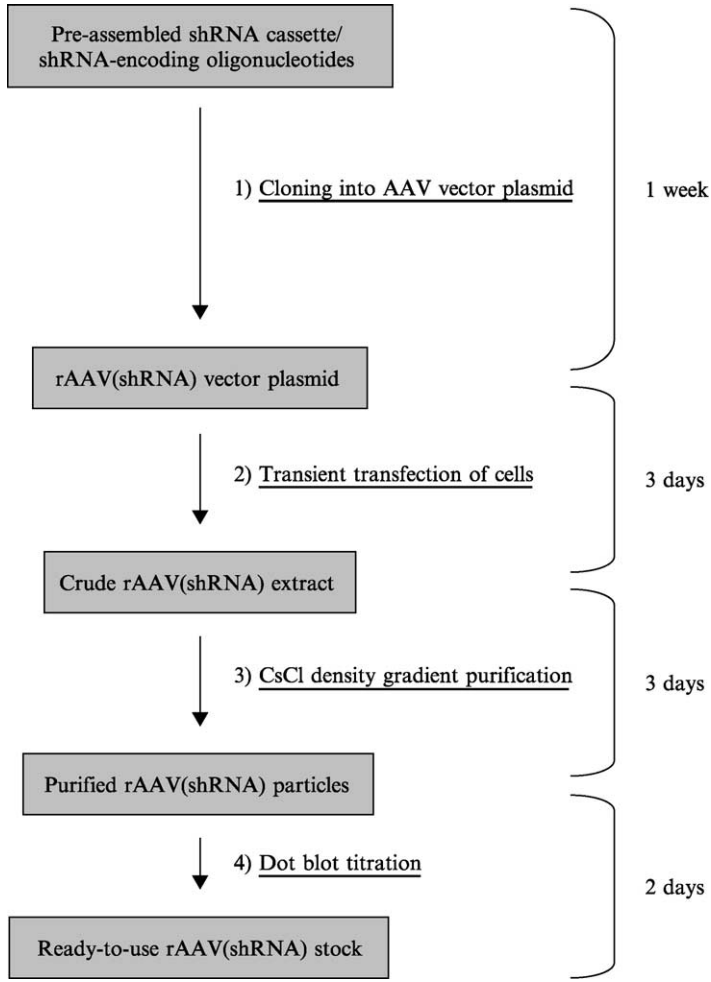

FIG. 3. Time frame for generation of shRNA-expressing AAV vector particles. Shown are the four different successive steps required to produce shRNA-encoding AAV particles, from cloning an shRNA cassette into an AAV vector plasmid to titrating the generated particles. Also shown are estimated times required for each step; the protocols described here take approximately 2 weeks to complete.

Step 1: Cloning shRNA-Encoding AAV Vector Plasmids. The following considerations are important.

SIZE. The generation of AAV particles expressing shRNAs follows the basic rules and protocols that have been established for conventional transgenes, using typical vector and helper plasmids. The only, yet very crucial, difference is that shRNA expression cassettes are typically significantly smaller than conventional inserts, comprising genes transcribed from RNA polymerase II promoters. Considering that an RNA polymerase III promoter such as H1 only spans approximately 100 bp and that the respective terminator sequence solely consists of a stretch of five thymidines, the total size of the entire shRNA expression cassette can be less than 150 bp. This creates the paradox that AAV, which has often been criticized as being too small to accommodate clinically relevant genes such as *cftr* (cystic fibrosis transmembrane conductance regulator gene), is suddenly much too large and requires a stuffer DNA to be added to the shRNA cassette to guarantee efficient vector genome encapsidation. This thought is based on a previous study by Dong *et al.* (1996), who quantitatively analyzed the DNA-packaging capacity of AAV-2 capsids and observed optimal encapsidation efficacies when the vector genomes had sizes between 4.1 and 4.9 kb (including the ITRs). Accordingly, the goal for cloning an shRNA-expressing AAV-2 vector plasmid should be to bring the size of the recombinant cassette up to a total of at least 3.8 kb (4.1 kb minus 2×145 bp for both ITRs), but not more than 4.6 kb.

Theoretically, any DNA sequence can be used as stuffer, but one particularly beneficial approach that has been reported repeatedly (e.g., Xia *et al.*, 2004) is to clone a second cassette expressing a fluorescence reporter gene under the control of an RNA polymerase II promoter. This provides the advantage that successfully transduced cells, presumably expressing the shRNA of choice, can be easily and rapidly monitored and quantitated. However, taking into account the relatively small size of typical fluorescence reporter genes of about 700 bp, generation of an AAV-2 vector plasmid comprising a total of at least 3.8 kb DNA might still require adding further sequences. We have devised a particular solution to this hurdle, which is discussed in the following section.

CHOICE OF VECTOR PLASMID. In principle, cloning an shRNA expression cassette into an AAV vector plasmid is a simple and efficient process. This is because most commonly available AAV vector constructs such as pTRUF3 (Zolotukhin *et al.*, 1996) typically contain several unique restriction enzyme recognition sites, allowing the straightforward insertion of foreign DNA, such as preassembled entire shRNA expression cassettes. This is achieved by using standard molecular techniques, which are not further described here. The plasmids are usually relatively small (<8 kb),

which additionally facilitates their handling and the cloning into the constructs. However, as mentioned previously, the size constraints of AAV capsids will likely require the addition of further sequences to the shRNA cassette, which can require more sophisticated strategies and thus complicate the cloning process.

To overcome this hurdle, we have generated a basic AAV-2/RNAi vector plasmid (Fig. 2A). This construct contains a 2.2-kb stuffer fragment from the *E. coli lacZ* gene as well as a 1.7-kb green fluorescence reporter gene (*gfp*) cassette, together comprising 3.9 kb. This is optimal for the insertion of very small shRNA expression cassettes such as the one mentioned previously with only 150 bp, but also leaves sufficient space for accomodating larger inserts of up to 700 bp without exceeding the AAV packaging limit. We have generated three alternative versions of this plasmid, which contain three different commonly used RNA polymerase III promoters to drive an shRNA, namely, U6, H1, and 7SK. Each plasmid version was engineered to contain unique restriction enzyme recognition sites, allowing the easy and straightforward insertion of customized shRNA sequences in the form of annealed oligonucleotides (see the protocol in this section). Likewise, these sites permit the replacement of any of the plasmid components, that is, the polymerase III promoter and the terminator sequence, the *lacZ* stuffer fragment, the fluorescence marker gene with its promoter, or even the entire recombinant insert (shRNA, *gfp*, and *lacZ*).

This particular plasmid design was chosen to provide maximum versatility and convenience by allowing users to rapidly clone shRNAs into AAV and express them under different RNA polymerase III promoters without having to worry about AAV size constraints. The constructs should be equally attractive for users who want to customize their shRNA-expressing AAV vector plasmid and who specifically intend to provide alternative marker genes or stuffer DNA. Further details of the plasmids and instructions for the insertion of shRNAs in the form of oligonucleotides are given in Fig. 2B.

Thus far, we have successfully exploited our constructs to clone shRNAs comprising 19- to 29-nt-long stem sequences. The respective oligonucleotides were purchased from IDT (Coralville, IA) at the lowest available amounts and degree of purity (standard desalting). Further purification, for example, by polyacrylamide gel electrophoresis, is often recommended for oligonucleotides longer than 50 nt (i.e., comprising stem sequences >21 nt: 2×21 plus the length of the hairpin and the Bbs I overhang), although we have not found this beneficial.

PROTOCOL. For annealing, the two oligonucleotides (1 μg each) are mixed in reaction buffer (50 m*M* NaCl, 10 m*M* Tris-HCl, 10 m*M* $MgCl_2$,

1 m*M* DTT, pH 7.9), heated to 95° for 5 min, and then allowed to cool down to room temperature for 30 min. In a subsequent ligation reaction, 2 μl of the annealed oligonucleotides are incubated with 50–100 ng of *Bbs* I-linearized AAV vector plasmid, using standard reaction conditions and ligation enzyme (e.g., T4 DNA ligase, NEB, Beverly, MA). The resulting recombinant shRNA-encoding AAV vector plasmids are selected and grown in DH10B bacteria, using ampicillin-containing LB medium.

Step 2: Producing shRNA-Expressing AAV Vector Particles by Transfection. The values given refer to transfection of 50 T225 flasks (Corning), which corresponds to approximately 1×10^9 cells (when grown to near confluency) and typically results in production of at least 10^{12} total AAV particles containing the recombinant shRNA sequence. The yields can vary dramatically (by more than one order of magnitude), depending on the particular AAV serotype being produced. In our hands, best yields are obtained with AAV serotype 8, followed by AAV-5, with both typically giving at least twofold higher particle titers than AAV-2.

PROTOCOL. To obtain 50 flasks for transfection, low-passage cells are first seeded into one T225 flask and grown until they reach confluency. The cells are then split and seeded into seven flasks, using standard cell culture technique, and then finally split one more time into 50 flasks. Therefore, old media from the seven flasks are discarded and cells in each flask are washed with 10 ml phosphate-buffered saline (PBS), followed by a 2-min incubation with 8 ml prewarmed 0.05% trypsin/EDTA solution. The flasks are then knocked to dislodge cells, and trypsin is neutralized by adding 8 ml DMEM per flask. The cells are resuspended, collected, and counted by a hemocytometer. Per new flask, 2.8×10^6 cells are plated and then incubated at 37° for 3 days until transfection (cells about 80% confluent at this point).

For transfection of all 50 flasks, a total plasmid DNA amount of 3.75 mg is used. Depending on the particular type of helper plasmids, this can be 1.25 mg each of an AAV and the adenoviral helper plasmid, as well as 1.25 mg of the shRNA-encoding AAV vector plasmid. Alternatively, if a combined AAV/Ad helper is used (Grimm *et al.*, 2003a), the vector plasmid is mixed with 2.5 mg of the hybrid helper construct. The plasmids are combined in a 500-ml disposable conical tube, followed by addition of 204 ml 0.3 *M* $CaCl_2$ and 204 ml 2× Hepes-buffered saline (HBS; 280 m*M* NaCl, 50 m*M* HEPES, 1.5 m*M* Na_2HPO_4, pH 7.1). Following vigorous shaking, 8 ml of the mixture (which will be slightly opaque) is aliquoted into each of the 50 flasks. Immediately before this step, 25 ml of the old medium in each flask is replaced with 25 ml fresh DMEM. The cells are then incubated with the DNA–HBS mixture for 5 h before the medium is aspirated and replaced with 40 ml fresh serum-free DMEM.

Following a 2-day incubation, the cells containing the shRNA-encoding rAAV particles are harvested by adding 0.5 ml of 0.5 *M* EDTA solution to each flask. After a 15-min incubation at room temperature, the flasks are knocked against a hard surface to lift cells off the surface. The medium containing the dislodged cells is then decanted into 500-ml conical tubes (cells from 10–12 flasks pooled into one tube), and cells are collected by a 15-min spin at 3500 rpm at room temperature. The resulting supernatants are aspirated and each cell pellet is resuspended in 30 ml of 50 m*M* Tris, 2 m*M* $MgCl_2$, pH 8.5. To release the AAV particles, the cells are then lysed by three freeze–thaw cycles (10 min at 37°, followed by 10 min in dry ice/ethanol bath) and subsequently incubated for 1 h with benzonase (endonuclease, Merck, Darmstadt, Germany) at 200 U/ml, to remove cellular genomic DNA. Following a 15-min spin at 3500 rpm, the supernatant is taken and diluted with 1 *M* $CaCl_2$ to a final concentration of 25 m*M*. This mixture is incubated on ice for 1 h and then centrifuged for 15 min at 9200 rpm in an SS34 rotor (Sorvall, Asheville, NC), before the resulting supernatant is transferred into a 50-ml tube. The total volume is estimated and one fourth of 40% PEG8000, 2.5 *M* NaCl solution is added to give a final concentration of 8% PEG8000. The mixture is incubated on ice for at least 3 h and then spun at 3500 rpm for 30 min. The pelleted rAAV particles are finally resuspended in 10 ml of 50 m*M* HEPES, 150 m*M* NaCl, 25 m*M* EDTA, pH 7.4; typically an overnight incubation is required to ensure that the viruses are completely dissolved.

Step 3: rAAV Particle Purification by Cesium Chloride (CsCl) Gradient Density Centrifugation. rAAV purification by CsCl gradient density centrifugation is a relatively time-consuming and cumbersome method as compared with newer protocols, such as those based on affinity chromatography. However, to our knowledge, it is the only available methodology that is applicable to all serotypes and capsid variants of AAV, which is why it was chosen to be reported here.

PROTOCOL. The solution containing the resuspended rAAV particles is vortexed and then centrifuged for 30 min at 3500 rpm. The supernatant is transferred into a new 50-ml tube, and the volume is adjusted to 24 ml with HEPES resuspension buffer. Following addition of 13.2 g CsCl, the refractive index (RI) of the solution is determined by a refractometer. The solution's RI is adjusted to 1.3710 by adding CsCl or resuspension buffer, and the solution is transferred into a 32.4-ml OptiSeal™ tube (Beckman, Fullerton, CA) and spun for 23 h in an ultracentrifuge at 45,000 rpm and 21°, using a 70Ti rotor (Beckman).

Subsequently, 0.5- to 1-ml fractions are collected through a hole poked into the bottom of the centrifuge tube and the RIs of each fraction are

determined. Fractions with an RI between 1.3711 and 1.3766 are pooled to a total volume of about 7 ml, and the RI of this final solution is again adjusted to 1.3710 with resuspension buffer. A second ultracentrifugation step is then carried out under the following conditions: 11.2-ml tubes and NVT65 rotor (both Beckman), 65,000 rpm, 21°, 5 h. Fractions are collected and those with an RI between 1.3711 and 1.3766 are pooled (total volume about 2.5 ml). The resulting rAAV-containing solution should then be instantly processed to the next purification step (see later); prolonged storage will result in a loss of particle infectivity.

To remove the CsCl and other contaminants still present, the rAAV preparation is finally subjected to a combined ultrafiltration–diafiltration process. Therefore, it is pumped through hollow fiber filters with a molecular cut-off of 100,000 Da (Amersham, Piscataway, NJ). The filtration is performed according to the manufacturer's instructions, using a constant back pressure of 14 psi and a pump speed of 385 rpm. Once the virus solution has been pumped into the filter unit, the cartridge is washed twice with PBS containing 5% sorbitol before the rAAV particles are eluted by operating the pump backward. The purified virus solution (volume 3–4 ml) is finally sterilized by filtration through a 0.22-μm filter.

Step 4: rAAV Particle Titration by Dot Blot. The protocol provided here will return a titer of shRNA-encoding AAV particles, which describes AAV virions containing the shRNA expression cassette. However, this titer does not necessarily correlate with the number of infectious particles, that is, virions that on transduction will actually express the shRNA. A more detailed discussion of this problem is beyond the scope of this chapter, but more information on the general correlation of DNA-containing infectious and total assembled rAAV particles can be found elsewhere (Grimm *et al.*, 1999).

PROTOCOL. To release viral DNA for quantification, the rAAV particles are first incubated with 5 U DNase I (Roche, Palo Alto, CA) in 100 μl of 20 m*M* Tris, 1 m*M* $MgCl_2$, pH 8.0, for 1 h at 37° to remove contaminating cellular DNA or unpackaged viral DNA that might still be present. Typically, several aliquots of the virus solution are digested, ranging from 0.5 to 0.05 μl. (For lower volumes the virus is prediluted in PBS.) The DNase digestion is followed by a 1-h incubation with proteinase K to break the viral capsids and release the encapsidated DNA. Therefore, 20 μl 10× proteinase K buffer (100 m*M* Tris, 100 m*M* EDTA, 5% SDS, pH 8.0) are added with 10 μl proteinase K (Invitrogen) and 70 μl H_2O. The mixture is vortexed and incubated at 55° for 1 h. The viral DNA is then purified by a DNA extractor kit (Wako, Japan), following the manufacturer's instructions, and is finally resuspended in 200 μl TE buffer.

To make a standard DNA dilution curve, 1 μg of the AAV vector plasmid used in the initial transfection is linearized by using a restriction enzyme that is unique for the particular construct. (See Fig. 2A for examples.) This is to account for the facts that the viral DNA is also linear and that linear and circular (i.e., uncut plasmid) DNA might bind to membranes (see later) with different efficiencies. The entire reaction is then diluted to a final DNA concentration of 5 ng/ml with TE buffer and subsequently further twofold diluted to 0.078 ng/ml (all dilutions in a total volume of 200 μl).

Viral DNA and standard samples are then denatured by adding 200 μl 2× alkaline solution (0.8 *M* NaOH, 20 m*M* EDTA), followed by vortexing and incubation at room temperature for 10 min. During the incubation, a 9 × 12 cm^2 Zeta-Probe membrane (Bio-Rad, Hercules, CA) is wetted in H_2O and assembled in a dot blot apparatus (Bio-Rad), following the manufacturer's instructions. The membrane is then equilibrated by pipetting 400 μl 1× alkaline solution into each well and applying vacuum until the wells are empty. The vacuum should be adjusted so that it takes between 30 and 60 sec for the wells to empty. The standard and the samples are added to the wells with the vacuum turned off and incubated on the membrane for 5 min. The vacuum is then applied again until the wells are empty, and the filter is finally washed with 400 μl 1× alkaline buffer per well. The apparatus is disassembled, the membrane is rinsed in 2× SSC and air dried on a filter paper, and the viral or standard DNA is cross-linked by using a Gene linker apparatus (program C1, Bio-Rad).

For detection and quantification of viral and standard DNAs, the membrane is then hybridized with a ^{32}P-labeled probe corresponding to a fragment of the rAAV vector plasmid (isolated by standard molecular techniques). It is first prehybridized for 30 min with 10 ml of 7% SDS, 0.25 *M* Na_2HPO_4, 1 m*M* EDTA, pH 7.2, by incubating at 65°. Then, 25–50 ng random-primed (Prime-It® Kit, Stratagene, La Jolla, CA) probe with a specific activity of 10^8–10^9 cpm/μg is denatured by heating to 95° for 5 min and then added to the membrane in 10 ml of fresh hybridization buffer. The membrane is then rotated with the probe overnight at 65° and the next day washed two times with 5% SDS, 40 m*M* Na_2HPO_4, 1 m*M* EDTA (20 min each), followed by two washes in 1% SDS, 40 m*M* Na_2HPO_4, 1 m*M* EDTA (20 min each). It is finally briefly rinsed in 2× SSC, air dried, and then exposed in a phospho imager cassette (Kodak, Rochester, NY). The image is captured and processed by using a Molecular Imager FX apparatus with respective software (Bio-Rad). A standard curve is generated, which then serves to calculate the rAAV titer, taking into account the initial sample dilutions.

Conclusions

The field of RNAi applications in mammalian systems *in vitro* and *in vivo* is still relatively young and in its early stages and so is the use of viral vectors for delivery and expression of shRNAs. It is, however, already becoming clear from the recent wealth of related publications that three classes of viruses—adenoviruses, retro/lentiviruses, and AAV—are emerging as most promising bases for vector development.

In particular, AAV, the focus of the present article, might represent an optimal bona fide system for delivery of shRNA expression cassettes, as AAV fulfils all the theoretical requirements of an ideal shRNA transfer system: it is generally very effective as a vehicle for transgene delivery *in vitro* and *in vivo*, as exemplified by research from the past 20 years; it is regarded as the safest of all known viral vector systems, with wild-type and recombinant AAV believed to be nonpathogenic, and rAAV particles by design being gutless and replication deficient; it has an enormously broad host range, including quiescent and dividing cells, with perhaps virtually every cell type being targetable because of the feasibilities to pseudotype and customize AAV genomes and capsids; it typically results in strong and persistent transgene expression in the infected cell, with a much lower risk of insertional mutagenesis than particularly retroviral vectors; and it can be manufactured easily and efficiently, allowing production of more than 10^{12} recombinant virus particles within 2 weeks.

Taking all these potential benefits of AAV as a vector for shRNA expression together, it is certain that this extremely promising system will be exploited, studied, and applied to a much greater extent in the near future. Our hope is that with the set of novel AAV vector plasmids we have presented here, which allow the rapid and easy cloning of customized shRNA sequences, we can help accelerate the pace of this exciting process.

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[24] Simple, Robust Strategies for Generating DNA-Directed RNA Interference Constructs

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Abstract

We describe two complementary strategies for preparing DNA-directed RNA interference (ddRNAi) constructs designed to express hpRNA. The first, oligonucleotide assembly (OA), uses a very simple annealing protocol to combine up to 20 short nucleotides. These are then cloned into appropriately designed restriction sites in expression vectors. OA can be used to prepare simple hairpin (hp)-expressing constructs, but we prefer to use the approach to generate longer constructs. The second strategy, long-range cloning (LRC), uses a novel adaptation of long-range PCR protocols. For LRC, entire vectors are amplified with primers that serve to introduce short sequences into plasmids at defined anchor sites during PCR. The LCR strategy has proven highly reliable in our hands for generating simple ddRNAi constructs. Moreover, LCR is likely to prove useful in many situations in which conventional cloning strategies might prove problematic. In combination, OA and LRC can greatly simplify the design and generation of many expression constructs, including constructs for ddRNAi.

Introduction

RNA interference (RNAi) is rapidly becoming a commonly used research tool (Berns *et al.*, 2004; Carmell and Hannon, 2004; Scherer and Rossi, 2003); moreover, the therapeutic potential of the technology is

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