Adeno-associated virus (AAV)-based vector systems are particularly attractive vehicles for clinical applications requiring long-term in vivo gene expression from post-mitotic tissues. AAV vectors have been shown to promote stable expression of a wide variety of transgenes in numerous tissues, including skeletal and cardiac muscle, liver, the central nervous system and retina (Rabinowitz & Samulski, 1998). Overt evidence of inflammation is either minimal or non-existent in target tissues immediately following AAV vector administration. Furthermore, cytotoxic T-lymphocyte responses are not normally elicited to transgene products delivered by AAV vectors, even when such proteins are foreign to the host (Jooss et al., 1997). AAV vectors are considered to be relatively safe because the parental virus is non-pathogenic and unable to replicate in the absence of a co-infecting helper virus. Additionally, current production methods have reduced the regeneration of replication competent wild-type AAV during vector production to undetectable levels (Allen et al., 1997). Finally, the robust protein capsid of AAV makes AAV vectors particularly amenable to existing production methods for protein pharmaceuticals (Gao et al., 2000) and confers upon them desirable drug stability characteristics.

AAV2, the parent virus from which the vector system is derived, is replication defective and requires co-infection of helper viruses to propagate. Adenovirus (Atchinson et al., 1965) and herpes virus (Buller et al., 1981) act as complete helpers and vaccinia virus (Schlehofer et al., 1986) acts as a partial helper. The set of adenoviral (type 2 or 5) genes that facilitate AAV2 propagation has been defined and consists of E1A, E1B55K, E1B19K, and protein IX) play in AAV vector production has not been clearly determined. E1 mutants were analysed for their ability to mediate AAV vector production in HeLa or KB cells, when cotransfected with plasmids encoding all other packaging functions. Disruption of E1A and E1B19K genes resulted in vector yield reduction by up to 10- and 100-fold, respectively, relative to the wild-type E1. Interruption of the E1B55K and protein IX genes had a modest effect on vector production. Interestingly, expression of anti-apoptotic E1B19K cellular homologues such as Bcl-2 or Bcl-x L fully complemented E1B19K mutants for AAV vector production. These findings may be valuable for the future development of packaging cell lines for AAV vector production.
single- and double-stranded forms of the AAV genome, the spliced forms of the rep proteins, and dramatically increases capsid protein production. Lastly, the VA RNAs inhibit the interferon-inducible eIF-2 protein kinase, thereby circumventing this cellular anti-viral mechanism from blocking viral protein translation (West et al., 1987).

With respect to E2A, E4orf6 and the VA RNAs, the helper gene requirement for AAV vector and virus production is identical. We and others, have shown that plasmids encoding these genes, when cotransfected into 293 cells along with plasmids encoding rep/cap and a vector, mediate higher levels of vector production than that produced by adenovirus infection (Xiao et al., 1998; Matsushita et al., 1998). This so-called ‘triple plasmid’ transfection method forms the basis of the current scale-up vector production effort at Avigen and has a respectable mean production efficiency of $1 \times 10^{13}$ vector genomes produced per 850 cm$^2$ roller bottle. A report was published describing a method for producing AAV vector in 293 cells using only E4orf6 as the helper gene (Allen et al., 2000). This method requires the use of a heterologous promoter to drive the capsid gene and is about 10-fold less productive than methods using a plasmid encoding all three adenoviral helper genes (unpublished data).

The genes of the E1 region have not been analysed for their contribution to AAV vector production. In this study, we have investigated the role of the $E1A$ and $E1B$ genes in AAV vector production by using a series of E1 mutant plasmids and cell lines that lack adenoviral genes. $E1A$ was required for efficient vector production. In contrast to the helper requirements for AAV production, our data indicated that $E1B19K$ gene greatly augmented vector production, however, $E1B55K$ gene did not.

The contributions of each of the component genes from the E1 region to AAV helper function was assessed by creating a set of plasmids with mutations in the $E1A$, $E1B19K$, $E1B55K$ or $protein\ IX$ genes and then testing them for their ability to support transfection-based AAV vector production. At least one truncation or one deletion mutation was made for each gene (Fig. 1).

For vector construction the plasmid pE1, which encodes the $E1A$, $E1B19K$, $E1B55K$ and $protein\ IX$ genes, was created from Ad2 DNA (Invitrogen). Briefly, the AflIII fragment (nt positions 142–5927) of Ad2 was cloned into the AflIII site of pBR322 (New England Biolabs) to generate pE1. pE1A-825stop was constructed by the insertion of an adapter (CCGGACTAATTAACTAGT), which includes a stop codon and an SpeI site, into the BspEII site of pE1. Similarly, pE1B19K-1912stop, pE1B55K-2243stop, pE1B19K-1912stop, pE1B55K-2243stop,
pE1B55K-2803stop and pE1B55K-3322stop were made by the insertion of oligonucleotides into the BstEII, BsrGI, HindIII and BglII sites of pE1, respectively. pE1A-del (616–933) has a deletion of a 318 bp segment (positions 616–933 in Ad2). pE1B19K-del (1772–1912) and pE1B55K-del (2803–3322) have the same deletions as dl337 (Pilder et al., 1984) and dl338 (Pilder et al., 1986), respectively, used by Samulski & Shenk (1988) to examine E1 helper function for AAV2 production. Briefly, pE1B19K-del (1772–1912) lacks sequences between positions 1772 and 1912, and pE1B55K-del (2803–3322) lacks sequences between positions 2803 and 3322. pProt.IX-3815stop was constructed by the insertion of oligonucleotides into a SacII site. pProt.IX-del (3654–3915) lacks a 262 bp segment (between positions 3654 and 3915 of Ad2).

The helper activities of the various E1 plasmids were assayed by cotransferring them with a plasmid encoding both an AAV CMVlacZ vector and rep/cap (pW4389LacZ), and a plasmid encoding the adenovirus-2 VA RNA, E2A and E4 regions (Pladeno5), into KB or HeLa cells, and then quantifying lacZ vector production as described previously (Matsushita et al., 1998). AAV vector was harvested 40 or 72 h after transfection and stocks were prepared by the freeze-thaw method. AAV vector production was quantified by titration of the vector stocks in 293 cells in the presence of adenovirus, followed by X-Gal staining and manual counting by light microscopy. For each experiment, all constructs were tested using triplicate production cultures, and all experiments were conducted at least three times, independently.

Elimination of the entire E1 region resulted in 2 (HeLa cells) to 3 log (KB cells) reduction in vector production relative to production in the presence of pE1, a plasmid encoding the entire E1 region (P < 0.01 by Student’s t-test) (Fig. 2a, b). Disruption of the E1A genes, whether by truncation or deletion, caused 1 (HeLa cells) to 1.5 log (KB cells) reduction in vector production (P < 0.01). Truncations or deletions in the E1B19K gene also resulted in substantial reduction in vector production, 1 log in HeLa cells and greater than 2 logs in KB cells (P < 0.01). The lesser severity of the E1B19K mutant in HeLa cells, relative to KB cells, may be due to the relatively high level of Bcl-2 expression in HeLa cells (Liang et al., 1995), or the human papilloma virus E6/E7 genes they harbour. The E6/E7 genes have been shown to facilitate some of the processes in AAV replication (Walz et al., 1997). In most cases, disruption of the E1B55K and protein IX genes had a modest effect on vector production in either HeLa or KB cells. Two constructs, pE1B55K-2243stop and pProt.IX-3815stop showed fivefold reduction in vector yield in KB cells but little reduction in HeLa cells.

Our results differ substantially from those of Samulski & Shenk (1988) who examined the effect of EIB adenovirus mutants on AAV2 production, DNA replication, and mRNA and protein expression. This group found that an E1B19K adenovirus-2 mutant (dl337) mediated efficient AAV production from HeLa cells transfected with a plasmid encoding an AAV wild-type provirus (pSM620) but that

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**Fig. 2.** Comparison of E1 mutant plasmids with respect to AAV helper function in KB (a) and HeLa cells (b) at 72 h after transfection, or in HeLa cells (c) at 40 h after the transfection. AAV lacZ vector was produced by the transfection of HeLa or KB cells with pW4389lacZ (encodes rep/cap and an AAV lacZ vector) and pladeno 5 (encodes the E2A, E4 and VA RNA regions), in the presence and absence of the indicated E1 plasmids. AAV vector production was assessed by titration of lacZ vector in 293 cells. pE1 (-) is identical to pBR322 without the expression cassette. Each bar represents the mean value obtained from triplicate cultures, and the error bar represents the standard deviation.
E1B55K (dl338) and E4orf6 (dl355) adenovirus mutants did not. AAV virion production was measured at a 40 h time point. The E1B55K and E4orf6 defects were caused by a delay in AAV mRNA accumulation that resulted in delays in viral DNA replication, capsid expression and ultimately virus production. AAV mRNA, DNA and capsid protein concentrations in cultures infected with E1B55K and E4orf6 mutants eventually reached levels seen in cultures infected by wild-type adenovirus but at longer time points, 72–96 h for adenovirus mutants compared with 24–40 h for wild-type adenovirus.

An important difference between our study and that of Samulski & Shenk (1988) was the timing of AAV/AAV vector harvest, 40 h in our study versus 72 h in theirs. Therefore, we examined a subset of the E1 region plasmids in transfection experiments using the same 40 h time point for vector harvest (Fig. 2c). The results were essentially similar to those at the 72 h time point and still differed from those produced by the adenovirus mutants. This observed difference in helper gene requirement may be attributable to technical factors associated with using virus infection or DNA transfection. A possible explanation for the conclusions reached by Samulski & Shenk (1988) might be the differences in the growth rates of the adenovirus mutants tested. The E1B55K mutant, dl338, was reported to grow inefficiently (100-fold reduced relative to wild-type) in HeLa cells (Pilder et al., 1986) while the E1B19K mutant, dl337, was reported to be less defective (about 10-fold reduced relative to wild-type) (Pilder et al., 1984). The lag in AAV mRNA, DNA and virus production seen with the E1B55K mutant may be simply because of a slow growing helper virus, resulting in low copy numbers of all of the adenovirus helper genes, and may not be directly due to the lack of the mutated gene. The observation that E1B19K is apparently not required for adenovirus mediated AAV production is harder to explain. It is tempting to speculate that the transfection-based production system benefits from additional anti-apoptotic activity provided by E1B19K. If this is true, this requirement does not appear to be cell-type or transfection-reagent specific (calcium phosphate and poly-cation-based transfection reagents both show an E1B19K effect, data not shown), and may have something to do with the adenoviral helper gene dose or kinetics of expression. Other differences between the two methods of identifying AAV helper function include: transfection method, the packaging of AAV virus versus a vector, and the use of replicating helper (AAV) versus non-replicating plasmid helpers. Full resolution of these issues will require further experimentation.

The adenovirus E1B19K gene, and its cellular homologues Bcl-2 and Bcl-xL, encode anti-apoptotic proteins that function by inhibiting proapoptotic Bcl-2 homologues, such as Bax and Bak, by forming inactive heterodimers with them. To determine whether other anti-apoptotic members of the Bcl-2 family could augment AAV vector production, plasmid vectors expressing the E1B19K, Bcl-2 or Bcl-xL gene products were tested for their ability to complement the vector production defect of the E1B19K deletion mutant, pE1B19K-del (1772–1912) (Fig. 3a). pTKPRMCS was assembled by the removal of a Renilla luciferase (RLuc) reporter gene from pRL-TK (Promega) (between the NheI and Xbal sites) and insertion of a multiple cloning site (between the Kpnl and Xbal sites) from pBluescript II (Stratagene). pTK-Bcl-2 and pTK-Bcl-xL were created by the insertion of human Bcl-2 and Bcl-xL cDNA sequences, respectively, into pTKPRMCS. pTK-E1B19K was constructed by the insertion of the E1B19K fragment into pTKPRMCS. As shown in Fig. 3(b), plasmids expressing E1B19K, Bcl-2 or Bcl-xL restored vector production of the E1B19K deletion mutant to levels equivalent to that produced by the wild-type pE1 plasmid. The use of the medium strength HSV-tk promoter to drive the expression
of the Bcl-2 homologues was essential for helper function. CMV-driven constructs produced low vector yields in a dominant fashion and caused a substantial increase in apoptosis (data not shown).

The fact that E1B19K mutants can be complemented by similarly anti-apoptotic cellular homologues such as Bcl-2 or Bcl-xL suggests a common mechanism, the inhibition of Bak/Bax-mediated apoptosis. Interestingly, no increase in DNA ladder formation is seen in HELa cells when transfected with E1B19K mutant plasmids relative to wild-type plasmids (data not shown). Consequently, the mechanism of vector production augmentation is not clear.

Current transfection-based AAV vector production methods are sufficient to commercially support gene therapy applications with large doses and small patient populations (e.g. haemophilia, other genetic diseases) or applications with small doses and large patient populations (e.g. Parkinson’s disease). Applications with large doses and large patient populations (e.g. heart failure) will be a challenge for transfection-based production methods that scale linearly. Consequently, the construction of a producer cell line that is both helper virus-free, and suspension culture-adaptable, is of great interest. This is a formidable task since many of the viral helper proteins are toxic to the cell either alone (e.g. E2A) or in combination with other helper functions (e.g. E4orf6 and E1B55K, E1A and rep). The task is further complicated by genes such as E1B19K that must be expressed in a rather precise manner. Packaging cell lines containing inducible E1 genes, along with the E2a, VA and E4 regions, and an integrated AAV vector have been produced but were found to suffer from relatively low vector yield and substantial production instability (Qiao et al., 2002). Both of these problems were likely due to, or exacerbated by, helper gene toxicity. Our data indicates that one source of toxicity, the inhibition of host mRNA nuclear export mediated by the E4orf6/E1B55K heterodimer, could be eliminated by not including the E1B55K gene in packaging cell lines.

Defining the minimum set of helper genes necessary for efficient vector production is the first step in creating suitable packaging cell lines for AAV vectors. Using our transfection-based assay, we define that set to be E1A, E1B19K, the VA RNAs, E2A and E4orf6 genes.

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