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Preventing Spontaneous Genetic Rearrangements in the Transgene Cassettes of Adenovirus Vectors

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Abstract

First-generation, E1/E3-deleted adenoviral vectors with diverse transgenes are produced routinely in laboratories worldwide for development of novel prophylactics and therapies for a variety of applications, including candidate vaccines against important infectious diseases, such as HIV/AIDS, tuberculosis, and malaria. Here, we show, for two different transgenes (both encoding malarial antigens) inserted at the E1 locus, that rare viruses containing a transgene-inactivating mutation exhibit a selective growth advantage during propagation in E1-complementing HEK293 cells, such that they rapidly become the major or sole species in the viral population. For one of these transgenes, we demonstrate that viral yield and cytopathic effect are enhanced by repression of transgene expression in the producer cell line, using the tetracycline repressor system. In addition to these transgene-inactivating mutations, one of which occurred during propagation of the pre-viral genomic clone in bacteria, and the other after viral reconstitution in HEK293 cells, we describe two other types of mutation, a small deletion and a gross rearranging duplication, in one of the transgenes studied. These were of uncertain origin, and the effects on transgene expression and viral growth were not fully characterized. We demonstrate that, together with minor protocol modifications, repression of transgene expression in HEK293 cells during viral propagation enables production of a genetically stable chimpanzee adenovirus vector expressing a malarial antigen which had previously been impossible to derive. These results have important implications for basic and pre-clinical studies using adenoviral vectors and for derivation of adenoviral vector products destined for large-scale amplification during biomanufacture.

Keywords

adenovirus; virus; vaccine; vector

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Conflict of interest: Okairòs Srl and the University of Oxford hold intellectual property related to adenovirus vaccine vectors.

Introduction

Interest in adenoviruses as vectors for gene delivery focused initially on gene therapy applications (reviewed by Zaiss et al., 2009), but from the early 1990s (Caravokyri et al., 1993; Jacobs et al., 1992; Xiang et al., 1996), recognition of their remarkable immunopotency has refocused interest on their use as recombinant vaccines (reviewed by Lasaro and Ertl, 2009), especially for development of desperately needed new prophylactic vaccines against the major global killers, HIV-AIDS (Haut and Ertl, 2009; Patterson et al., 2009), malaria (Hill et al., 2010; Limbach and Richie, 2009), and tuberculosis (McShane and Hill, 2005; Xing and Charters, 2007).

The high prevalence of neutralizing serum antibody against the most widely studied vector, based on human adenovirus type 5 (HAdV-5), and other serotypes endemic in the human population worldwide has led to the use of rare serotypes (Abbink et al., 2007) or viruses of non-human origin, most notably chimpanzee adenoviruses (Ersching et al., 2010). These have much lower seroprevalence in humans, and are not cross-neutralized, so can elude the dampening of vaccine potency by pre-existing anti-adenoviral immunity (Roberts et al., 2006). Vaccination regimens employing simian adenovirus vectors induce potent cellular and humoral immune responses in non-human primates (Capone et al., 2010; Draper et al., 2010; Santra et al., 2009) and first-time-in-man phase I/IIa clinical trials of liver- and blood-stage malaria vaccines using a chimpanzee adenovirus 63 (ChAd63) vector (Sheehy et al., 2011) and a phase I trial of a hepatitis C vaccine using a ChAd3 vector, are currently underway in Oxford, UK (<http://clinicaltrials.gov/ct2/results?term=adenovirus+oxford>).

In a “first generation” adenoviral vector, the essential E1 locus is deleted and complemented in trans in a producer cell line, such as HEK293, usually in combination with deletion of the non-essential E3 locus for further attenuation and to allow insertion of longer transgenes. The production of such vectors has become routine, with several commercial kits available for HAdV-5 and other commonly used serotypes. The apparent ease of generation belies the complexity of the underlying biology: it is all too easy to overlook the possibility that one may be impairing viral growth by over-expression of certain exogenous transgenes. Indeed, each new vector–transgene combination could be regarded as a novel semi-synthetic life form, undergoing artificial selection in laboratory culture.

Here, we provide a compelling demonstration of strange, aberrant genetic structures of unpredictable nature that were encountered in production of two ChAd63 adenoviral vectors carrying transgenic cassettes for expression of the *Plasmodium falciparum* antigens Pfs230 or circumsporozoite (CS) protein. Evidence-based strategies to minimize the generation of mutants ab initio, by modification of the vector design and production protocol, are shown to allow isolation of the correct desired viruses. Since the underlying mutagenic mechanisms are generic and pertain to all adenoviral vectors, and possibly to any transgene, these findings have the potential to enhance the interpretability and robustness of basic and pre-clinical research on adenoviral vectors, as well as to facilitate and improve production bioprocesses for their manufacture at clinical trial or product scale—the feasibility of which is critical for deployment.

Materials and Methods

Generation of Recombinant ChAd63 Pre-Viral Clones

A genomic clone of ChAd63 was provided by Okairòs S.A., Rome, Italy. The plasmid (pChAd63) comprises the viral genome, with the E1 and E3 loci deleted, and the E4 locus replaced with E4ORF6 from HAdV-5, in a pUC vector flanked with unique *PmeI* sites. Transgenic cassettes for insertion into the E1 locus were constructed using standard cloning techniques. For insertion by gap repair (Chartier et al., 1996), BJ5183 cells were electroporated with pChAd63 linearized via a unique *AsSI* restriction site at the former E1 locus, together with a template plasmid containing the cassette for insertion and flanking regions for recombination. Clones were screened by restriction digest and candidates were transferred into DH5 α cells for amplification and verification. For insertion by Gateway cloning (www.invitrogen.com/gateway/), *AttR* sites and appropriate selectable markers were introduced into pChAd63 at the *AsSI* site by standard restriction digestion and ligation to create pChAd63-DEST in *ccdB*-survival cells (Invitrogen Corp, Carlsbad, CA). LR clonase (Invitrogen) was utilized to insert cassettes cloned conventionally into ENTR plasmids containing flanking *AttL* sites, prior to verification and propagation in DH5 α cells.

Isolation, Amplification and Purification of Viruses

For viral rescue, pChAd63 was digested with *PmeI* to liberate the genomic termini and transfected into producer cells using Lipofectamine (Invitrogen). HEK293 cells were obtained from ECACC and cultured according to standard practices. HEK293-TetR cells were either T-Rex HEK293 cells (Invitrogen) or a similar HEK293-based cell line with traceable origin suitable for manufacturing, Procell-92, provided by Okairòs S. A., Rome, Italy (S. Colloca et al., manuscript in preparation). Both were cultured according to the suppliers' protocols. Viruses were amplified in HyperFlasks (Corning Inc, Corning, NY), harvested by triple freeze-thaw of the cell pellet in the presence of benzonase (250 U/mL) and purified on a discontinuous CsCl gradient (1.25–1.35 g/mL) spun for 2 h at 110,000g and then by isopycnic ultracentrifugation in 1.35 g/mL CsCl for 18 h at 160,000g. The harvested bands were dialyzed against 10 mM Tris, pH 7.5, containing 7.5% sucrose for storage at –80°C. Viral particles were quantified by UV spectrophotometry in the presence of 0.1% SDS. Viral genomic DNA for restriction endonuclease analysis was prepared by proteinase K digestion and extraction with phenol–chloroform.

Quantitative PCR Analysis

The following primers and hydrolysis probes were utilized in conjunction with Gene Expression Master Mix (ABI) and an Applied Biosystems Step One Plus thermal cycler: AmpR forward primer: 5'-GTACGGTGGGAGGTCTATATAAGCA-3'; AmpR reverse primer: 5'-GGTGTCTTCTATGGAGGTCAAACA-3'; AmpR probe FAM-5'-AGCTCGTTTGTGAACCGTCAGATCGCC-3'-TAMRA; CMV forward primer: 5'-TGCTGCCATAACCATGAGTGATAAC-3'; CMV reverse primer: 5'-GTCCTCCGATCGTTGTCAGA-3'; CMV probe: FAM-5'-CTGCGGCCAACTTACT-3'-TAMRA. Reactions were prepared in a microbiological safety cabinet to prevent contamination with ampicillin-resistant plasmids, which are also present in some commercial polymerase mixes. Phenol-purified viral genomic DNA corresponding to 5 ×

10^7 viral particles was diluted into 5 μ L water per reaction and the copy number of each template was determined by reference to a standard curve prepared using an AmpR plasmid containing CMV promoter. The standard curves were linear from 50 to 5×10^8 copies and samples were analyzed once or twice in triplicate.

Protein Expression

Circumsporozoite protein was detected in fixed and permeabilized ChAd63-CSN-infected 293 cells with 2A10 monoclonal antibody (Malaria Research and Reference Reagent Resource Centre, Manassas, VA) and Alexa-488 conjugated secondary antibody (Invitrogen) and the mean fluorescence intensity of the infected population was quantified by flow cytometry. CSN protein in transduced A549 cells was detected via a capture ELISA. A549 cells were inoculated with 1 infectious unit per cell of ChAd63-CSN and lysed 48 h later in 0.2% Triton X-100 in PBS containing protease inhibitor cocktail (Perbio, Thermo Fisher Scientific Inc, Waltham, MA). ELISA plates were coated with 1 μ g/well of purified 2A10 monoclonal antibody and blocked before application of serially diluted lysates. Bound CSN protein was detected using polyclonal rabbit anti-CSP (Malaria Research and Reference Reagent Resource Centre), alkaline phosphatase conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) and development with *p*-nitrophenyl phosphate. Absorbance was measured at 405 nm and wells lying in the linear assay range (corresponding to a 1:125 dilution of lysate) were selected.

Immunogenicity

For mouse immunogenicity, CsCl-banded viruses were titred using a hexon immunostaining kit (Cell Biolabs Inc, San Diego, CA, USA) modified for 96-well plates and Balb/c mice were immunized i.m. with 10^8 infectious units in accordance with the UK Animals (Scientific Procedures) Act 1986. Antigen-specific CD8⁺ T-cell responses were measured by ELISpot assay of splenocytes 2 weeks post-vaccination, as previously described (Sridhar et al., 2008), using ex vivo restimulation with a pool of synthetic overlapping oligopeptides corresponding to the antigen protein sequence.

Results

Design and Construction of ChAd63-CSN

A widely used system, comprising a cytomegalovirus (CMV) promoter, the open reading frame (ORF), and a polyadenylation signal (polyA), inserted into the former E1 locus of the adenoviral vector genome as previously described (Sridhar et al., 2008), was employed for expression of *Plasmodium falciparum* CS protein in the ChAd63 vector. The promoter was the wild-type CMV major immediate-early promoter-enhancer, which contains an exon, intron, and splicing sites in addition to the enhancer region, herein referred to as the “long” CMV promoter. The ORF, designated “CSN,” was a synthetic, codon-optimized version of CS lacking the C-terminal 9 amino acids to inactivate the signal sequence for the GPI anchor; and the polyA was the widely used bovine growth hormone (BGH) sequence. This transgene cassette was inserted into a pUC-based genomic clone of E1/E3-deleted ChAd63 by plasmid gap repair in BJ5183 *E. coli*, and the virus was rescued by transfection of the resulting plasmid (verified by restriction analysis and sequencing of homology arms and

insert) into HEK293 cells following digestion with *PmeI* to excise the pUC backbone and liberate the genomic termini, all in accordance with standard protocols for adenoviral vectors.

A Bacterially Derived Mutation That Inactivated the Transgene of ChAd63-CSN

Prior to initiation of manufacture of an adenovirus vector for human trials according to current good manufacturing practise (cGMP), it is typically necessary to demonstrate viral genetic stability using the assay recommended by the US Food and Drug Administration (FDA), comprising genomic restriction endonuclease analysis after prolonged propagation in the producer cell line. Having encountered no problems with three other ChAd63 vectors expressing *P. falciparum* antigens (apical merozoite antigen 1, merozoite surface protein 1, and thrombospondin-related adhesion protein; Capone et al., 2010; Draper et al., 2010; Sheehy et al., 2011), we were disappointed to observe anomalous results in restriction digestion analyses of ChAd63-CSN following 10 serial passages of the virus in HEK293 cells. Sequencing of the viral DNA revealed that a bacterial transposon, or insertion sequence, called IS150 (GenBank: X07037.1), was present in the transgene, such that the CSN ORF was interrupted (Fig. 1A). This accounted for the presence of aberrant bands of increased size in the passage 10 restriction digests (Fig. 1B), which were not observed prior to serial passage but represented the sole detectable species after passage. Despite its restriction digests showing the expected patterns, we were able to detect the IS150 insertion in the un-passaged ChAd63-CSN by PCR as a minor species (data not shown). Since the IS150-carrying virus did not express CSN protein (data not shown), we concluded that a virus derived from a very rare contaminant in the transfected bacterial plasmid had exhibited a growth advantage during amplification.

The virus failed the genetic stability assay not because of viral genetic instability per se, but because of low-level contamination with a bacterially derived mutant virus. The insertion of the transgene cassette into the ChAd63 genomic clone was performed using gap repair in recombination-competent BJ5183 *E. coli*, but the resulting pre-viral plasmid was subsequently transferred into the DH5 α strain. Since the IS150-inactivated mutant was a very minor contaminant, we concluded that the transposon mutagenesis had occurred during plasmid amplification in the DH5 α bacteria. Insertion sequences have been reported as the major mutagenic mechanism in the related strain DH10B, another commonly used K-12-derived *E. coli* cloning strain, and contamination of genomic library clones with insertion sequences is by no means uncommon (Durfee et al., 2008).

Isolation of Mutant ChAd63-CSN With Deletion at the CMV Promoter Splice Acceptor Site

The failed genetic stability assay was performed using a pooled viral isolate, so we attempted to obtain plaque-picked isolates free of the IS150 insertion mutant. Of 24 plaques analyzed by PCR, 8 contained IS150 mutations. Two plaques that were negative in PCR assays for IS150 were taken forward for a repeat genetic stability assay. In one of these isolates (but not the other) we identified a 66 bp deletion in the region of the predicted splice acceptor site of the CMV promoter (Fig. 2A), accounting for the aberrant size of a band in the genomic restriction digest (Fig. 2B). In this case, the mutation was present even before the 10 serial passages (i.e., in the original plaque) and remained unchanged after this

amplification. Although the virus did express CSN protein when analyzed by Western blotting (data not shown), we did not attempt to quantify the effect of this splice site deletion on protein expression levels compared to the wild-type promoter. Unlike the IS150 insertion, which could only be bacterial in origin, this 66 bp deletion mutation could have occurred at either the bacterial or viral stage: we have not determined experimentally which was the case.

Isolation of Mutant ChAd63 Carrying a Duplication of Part of the Transgene Cassette

Despite lacking the 66 bp deletion, the second viral plaque isolate had a different problem, and one that was shared by two other plaque-derived isolates subsequently examined (Fig. 3A). This was a duplication of the CMV promoter and part of the CSN ORF, comprising two complete copies of the promoter and a mutated copy of CSN (vCSN) fused to the 5' end of the duplicated promoter, resulting in a C-terminal nonsense extension of the predicted translation product, in addition to a correct CSN ORF further downstream. After deducing this arrangement following sequencing of different restriction fragments, culminating in obtaining sequence across the junction, we were able to account for the aberrant bands observed by genomic restriction endonuclease analysis (Fig. 3B). The phenomenon is most clearly discernable in the digest with *Sna*BI (Fig. 3), which has a unique site in the ChAd63-CSN CMV promoter that is duplicated in the mutated virus. As with the 66 bp deletion, this duplication was also present prior to the genetic stability assay, and was stable over 10 passages (data not shown). Although we once again did not perform a quantitative analysis in comparison to wild-type, the duplication did not abrogate protein expression, as detected by Western blotting. Whether this protein corresponded to the downstream CSN ORF or the upstream vCSN mutated ORF, or both (Fig. 3A), it is not clear, since the predicted molecular weight of vCSN is almost identical to that of the unmutated protein; and also the vCSN ORF lacks its own polyA signal, so may not be expressed. As in the case of the 66 bp deletion, this duplication mutant could have arisen either during or after manipulation and amplification in *E. coli*, but we have not determined experimentally which was the case.

Out of 24 ChAd63-CSN plaque picks, 8 contained IS150; 1 carried the 66 bp deletion; 5 had the duplication; and the remaining 10 either displayed incorrect band sizes of PCR amplicons spanning the transgene region, or lacked protein expression on Western blots, and were not characterized further. Thus, none of these isolates was suitable for generation of ChAd63-CSN manufacturing seed stocks, and indeed a substantial proportion (greater than one-third) lacked a functional transgene. Despite this, the lab-scale pre-clinical material made by straightforward amplification of the virus after rescue and banding on CsCl gradients appeared correct, both in PCR assays and by restriction digest (data not shown), though presumably must have contained very low-level contamination with these aberrant species. These minor contaminants came to dominate the population either after serial passage (as in the case of IS150) or after plaque-picking (as in the case of IS150, the 66 bp deletion, and the duplication), suggesting that they possessed a selective advantage.

Virally Mediated Transgene Inactivation by Insertion of Co-Transfected DNA Into ChAd63

For a separate project, we constructed a ChAd63 vector expressing part of the malarial candidate transmission-blocking antigen Pfs230 (Williamson, 2003), using the same

expression cassette. When produced in HEK293 cells at lab scale ($\sim 10^{12}$ viral particles), a mutant viral species was detected in routine pre-clinical PCR quality control assays, following only the amplification required for production of material for mouse vaccination (in contrast to the equivalent degree of propagation of ChAd63-CSN used in Fig. 1, passage 1). This virus carried a deletion of most of the CMV promoter and nearly all of the Pfs230 ORF, achieved by way of insertion of the entire pUC plasmid backbone (Fig. 4A) that had been previously excised by restriction digestion to liberate the viral genomic termini for transfection. The sequence at each junction contained part of one of the two *PmeI* sites employed for this excision. Although not the major amplicon, the smaller PCR product representing the mutant virus increased in abundance following CsCl gradient banding compared to pre-amplification bulk lysate (Fig. 4B), again suggesting a powerful growth disadvantage of the intended viral construct, similar to outgrowth of the IS150-inactivated ChAd63-CSN.

In this case, the mutation is certainly of viral origin, unlike the IS150 insertion (certainly bacterial) and the 66 bp deletion and the duplication in ChAd63-CSN (unknown whether bacterial or viral). Since the inserted sequence corresponded exactly to the fragment excised from the purified pre-viral plasmid prior to viral rescue, only a viral recombination event could explain its insertion into the transgene. This demonstrates unequivocally that mutagenic events can occur as a result of viral recombination, as well as by instability in *E. coli*. Furthermore, since genomic excision is a critical step in adenoviral rescue, and since the observed recombination was non-homologous, the phenomenon may represent a potential problem for all viruses derived by this method (including ChAd63-CSN).

Repression of CSN Gene Expression Increases ChAd63-CSN Growth Rate

On the basis that the problems we encountered with ChAd63-CSN were attributable to a selective disadvantage associated with viral over-expression of CSN protein, regardless of whether the mutation was of bacterial or viral origin, we generated new versions of the vector transgene cassette incorporating tetracycline operator (TetO) sequences downstream of the CMV promoter TATA box to enable repression of gene expression in HEK293 cell lines carrying a gene encoding the tetracycline repressor protein (HEK293-TetR). For these new constructs, we used site-specific recombination (Gateway cloning) to insert the transgene cassette into a suitably modified ChAd63 genomic clone, instead of gap repair in BJ5183 cells.

We created two TetR-repressible ChAd63-CSN constructs, one based on the “long” or wild-type, intron-containing CMV promoter (as used up until this point) and one on the “short” intronless, non-splicing version of the promoter (Fig. 5A). The productivity of viral infections of ChAd63-CSN employing either of these repressible promoters was improved by 10-fold or more in HEK293-TetR cells compared to HEK293 cells (Fig. 5B), and was associated with an earlier onset of cytopathic effect (Fig. 5C). The levels of CSN protein were substantially reduced in HEK293-TetR cells (Fig. 6B), confirming that CSN over-expression is indeed associated with impaired viral growth. These results provide an explanation for the outgrowth of non-expressing ChAd63 during bulk propagation and the isolation of incorrect viruses by plaque-picking.

Comparative Expression and Immunogenicity of “Short” and “Long” CMV Promoters

The mutation observed at the CMV promoter splice site (Fig. 2) led us to favour subsequent use of the “short” variant, lacking any splicing, for ChAd63-CSN. The “short” intronless CMV promoter has, however, been reported to drive reduced protein expression in cells infected with adenoviral vectors (Hermening et al., 2004; Sridhar et al., 2008), and to elicit reduced immunogenicity in mice compared to the “long” spliced version (Sridhar et al., 2008). We therefore compared the murine immunogenicity (Fig. 6A) of ChAd63-CSN vectors employing either “short” or “long” CMV promoters. In accordance with our previous work (Sridhar et al., 2008), the virus with the “long” promoter elicited somewhat (1.4-fold) higher specific IFN- γ ELISpot responses in vaccinated mice (Fig. 6A), though the difference was not statistically significant ($P = 0.09$, t -test). The protein expression levels detected by antibody labeling and flow cytometry appeared virtually identical in HEK293 cells (Fig. 6B). Since the viruses replicate in 293 cells, increasing copy number of the transgene and potentially concealing differential promoter activity, we compared CSN protein expression in A549 cells, which do not support viral replication, using a more sensitive capture ELISA method to detect the overall lower protein levels. Under these conditions, the “short” promoter yielded approximately twofold less CSN protein than the “long” promoter.

Derivation of a ChAd63-CSN Construct That Remains Faithful to Its Design

On the basis of these results, we proceeded with a “short,” TetO-containing repressible promoter version of ChAd63-CSN in a second attempt to generate input virus for clinical manufacturing using HEK293-TetR cells for viral rescue and propagation. As well as reducing the selective advantage of potential mutant clones by transgene repression, we made two minor modifications to the method of ChAd63-CSN derivation in order to circumvent previously encountered issues.

We used limiting dilution instead of plaque picking in order to avoid any bias in microscopical selection of viruses exhibiting enhanced CPE as a result of decreased CSN expression. Repression of CSN expression accelerated CPE (Fig. 5C), and previously only incorrect plaques, over one-third of which were non-expressors, were isolated from bulk input material that contained only minor incorrect contaminants. Two limiting dilution isolates prepared in HEK293-TetR cells were selected as candidate cGMP input seed stocks and characterized further.

We band-purified the excised viral genome prior to rescue in order to remove the template for recombination events similar to the observed inactivation of the ChAd63-Pfs230 transgene by the non-viral plasmid fragment (Fig. 4). Although we had not observed such a mutation in ChAd63-CSN, we were concerned, for the reasons given above, that this could be a generic phenomenon. Additionally, it is a requirement of cGMP to demonstrate the absence of the *bla* gene (AmpR) of the plasmid backbone from the final product. We therefore utilized a real-time PCR assay to quantify the copy number of AmpR relative to that of the CMV promoter in the cesium-banded viruses before and after 10-fold serial passage (Fig. 4C). Surprisingly, despite pre-viral plasmid band excision and benzonase digestion during cell lysis, we could detect significant AmpR contamination after limiting

dilution but prior to passage, of 47 ppm (95% CI: 12–180 ppm) for isolate A and 163 ppm (46–578 ppm) for isolate B. Following passage, these figures fell to 7.1 ppm (2.6–19.7 ppm) for isolate A and 4.4 ppm (0.5–38.6 ppm) for isolate B. These figures are close to the limit of sensitivity of the assay, which cannot detect below ~1 ppm AmpR/CMV promoter, corresponding to ~50 copies (~50 ag) of AmpR amplicon template DNA, probably owing to the ubiquitous usage of AmpR plasmids in Molecular Biology Laboratories. Therefore, there was no evidence of propagation or detectable presence post-passage of a virus carrying the pUC plasmid backbone, as was observed for ChAd63-Pfs230 (above).

The two new, band-excised, limiting-diluted isolates of ChAd63-CSN containing the “short,” TetO-containing CMV promoter and prepared in HEK293-TetR cells were subjected to a genetic stability assay. Neither displayed any alteration in three different viral genomic restriction digests (two of which are shown in Fig. 6C) or CSN protein expression by Western blot (data not shown) over 10 serial passages in either HEK293 cells or HEK293-TetR cells. Sequencing of PCR products amplified from viral genomic DNA and spanning the transgene and the immediate flanking regions confirmed that the sequence of this region was correct after viral passage in both cell lines. Thus, by circumventing putative mutational mechanisms and reducing the selective advantage conferred by mutation, the newly derived ChAd63-CSN was shown to be suitable for large-scale amplification and clinical manufacture even without repression of transgene expression in standard HEK293 cells.

Discussion

During derivation of E1/E3-deleted ChAd63 vectors with CMV promoter driven expression cassettes inserted at the E1 locus, we encountered four aberrant viruses carrying different mutations in the transgene (Figs. 1–4), three with ChAd63-CSN during experiments specifically designed to evaluate genetic stability prior to manufacture for clinical trials (Figs. 1–3), and one with ChAd63-Pfs230 in a normal lab-scale preparation (Fig. 4). This was unexpected, since we have successfully generated >100 recombinant adenoviral vectors at small scale, and did not encounter any such problems in derivation of three previous ChAd63 vectors carrying other malarial antigens for clinical-scale manufacture using the same protocols (Capone et al., 2010; Draper et al., 2010; Sheehy et al., 2011). Furthermore, HAdV-5 and HAdV-35 based vectors containing CS transgenes are currently in early-phase clinical development (Limbach and Richie, 2009; Rodrigues et al., 1997).

Two of these four mutations involved insertional inactivation of the ChAd63 transgene, either by a bacterial transposon, IS150 (Fig. 1); or by the co-transfected DNA fragment corresponding to the plasmid backbone that was excised enzymatically prior to viral rescue (Fig. 4). In both cases, the mutant virus was initially a minor contaminant, but exhibited a selective growth advantage in HEK293 cells, and came to dominate the viral population over time. For ChAd63-CSN, we show using the TetR system that repression of transgene expression, confirmed at the protein level, is associated with dramatically increased viral yield and accelerated CPE, consistent with the observed outgrowth of mutant non-expressors.

We also observed a deletion of the “long” CMV promoter splice acceptor site (Fig. 2), and a partial duplication/rearrangement of the transgene (Fig. 3). The effects, if any, upon transgene product levels or viral growth were not investigated: the only indication of any phenotype is that they were over-represented in plaque isolates of ChAd63-CSN compared to the input. Speculatively, given the dramatic effect of CSN repression on CPE (Fig. 5C), the repeated isolation of these mutant viruses by plaque picking could be the result of a decrease in CSN expression caused by loss of the splice site or by readthrough transcription in the partially duplicated transgene, though we have not investigated these possibilities or shown that CSN protein levels are reduced. The origin of these mutations is also unclear: one might speculate that they were of viral origin, since transposon-mediated and point mutations have been reported to be the dominant mutagenic mechanisms in *E. coli* (Durfee et al., 2008), but we have not attempted to establish whether they occurred at the bacterial or viral stage of derivation.

In a subsequent, successful attempt to generate a correct isolate of ChAd63-CSN, we simultaneously adopted five modifications to the procedure used for viral derivation, based on circumventing the problems previously encountered, described above. Although we worked only with ChAd63 in these studies, and with only two transgenes (and only one in any detail), it is likely that similar mutational mechanisms could operate in other adenoviral species or types, and in other transgenes. We will therefore discuss the possible generalized utility of each type of intervention to adenoviral vector derivation and production, bearing in mind that we have not tested them individually.

First, we used site-specific in vitro recombination (Gateway cloning) instead of homologous recombination in BJ5183 *E. coli* to insert the transgene cassette into the viral genomic clone. Although we concluded that the transposon insertion did not occur in BJ5183 cells (see above), we wished to avoid their use, given the lack of knowledge about the mechanism of recombination in the strain (Takahashi et al., 2003) and the undetermined provenance of two of the mutations observed. Whether there is any difference in the fidelity of these two methodologies has not been evaluated in this work. We did not take any steps to mitigate insertional transposon mutagenesis in *E. coli*. In the DH10B strain, this problem is by no means uncommon, and indeed IS150 has been identified as the most frequent culprit (Durfee et al., 2008). The *E. coli* strain MDS42, which lacks all insertion sequences (Posfai et al., 2006), and has been shown to allow maintenance of clones that are otherwise reproducibly mutated (Umenhoffer et al., 2010), could be beneficial for propagation of adenoviral genomic clones.

Second, with the rationale of preventing phenomena similar to that shown in Figure 4, the band corresponding to the viral genome was excised and purified from an agarose gel, prior to transfection of the pre-viral clone, so that only a single predominant nucleic acid species, rather than a 1:1 mixture, was introduced into eukaryotic cells for viral rescue. A quantitative PCR assay was developed to demonstrate the absence (within its detection limits) of plasmid backbone derived sequence from the viral genome after passage. The use of a “self-excising” system for viral rescue (Gao et al., 2003) may be an alternative possibility. Since we have only ever observed this type of mutation in ChAd63-Pfs230 (Fig. 4), we have not been able to evaluate whether this precaution confers any generic benefit.

Third, we employed limiting dilution rather than plaquepicking, because we were concerned that microscopical selection of ChAd63-CSN plaques with florid CPE might account for the isolation only of mutated viruses by this method, given the observed acceleration of CPE observed when CSN expression was repressed, although we have no direct evidence with mutant viruses to support this notion. Furthermore, since we performed the limiting dilution in HEK293-TetR cells, we cannot conclude that this method is any more likely to yield non-mutant isolates, though it is certainly quicker and easier.

Fourth, we used the “short” CMV promoter, lacking the intron, instead of the “long,” spliced promoter, to drive transcription of the CSN ORF. The disadvantage of this approach is that, in accordance with published work (Hermening et al., 2004; Sridhar et al., 2008), ChAd63-CSN carrying the “long” promoter elicited marginally higher frequencies of antigen-specific T cells in mice (though this was not statistically significant) and directed about twofold greater protein expression in non-permissive cells in vitro. The motivation for this change was that, as it lacks the intron and splice sites anyway, the “short” promoter would at not be susceptible to the 66 bp deletion mutant encompassing the splice acceptor site observed with the “long” promoter (Fig. 4). The questions of whether these two variants of the CMV promoter are differentially susceptible to mutagenesis in adenoviral vectors of the nature described here, and whether any such phenomenon is transgene-specific, remain to be addressed, but may be important for reliable manufacture of optimally immunogenic and genetically stable adenoviral vectors.

Finally, we introduced TetO sites into the CMV promoter of ChAd63-CSN and derived the virus in HEK293-TetR cells to repress transgene expression during production. This reduced CSN protein levels by >10-fold, improved viral yield by a similar factor, and accelerated CPE. This dramatic phenotype indicates that Tet-repression (or a similar system) is an important intervention for improving amplification fidelity and yield of adenoviral vectors, and has the added benefit of obviating the need to empirically determine optimal viral culture conditions for each construct (Stanton et al., 2008). The mechanism(s) by which certain transgene products interfere with the viral and/or cellular processes involved in viral replication remain to be determined.

Generation of infectious virus from plasmid DNA is extremely inefficient: the vast majority of transfected cells do not go on to yield infectious progeny. Transfection of HEK293 cells with 2 µg of excised pre-viral plasmid DNA, corresponding to $\sim 5 \times 10^{10}$ genome copies, produces only ~10 adenoviral plaques, with an approximately 10-fold improvement via a “self-excising” rescue system (Gao et al., 2003). Anecdotal evidence can be found in the literature suggesting that some transgenes cannot be rescued into adenoviral vectors at all (Youil et al., 2003), and one of the advantages of cGMP-grade HEK293-TetR cells (Procell 92) is that they enable rescue of adenoviral vectors that cannot be generated in the absence of repression (S. Colloca et al., manuscript in preparation).

The data presented here show that, in cases where transgene expression impairs adenoviral vector propagation, there is a substantial possibility of producing vectors with transgene mutations that confer an advantageous growth phenotype compared to the desired virus. We have observed mutations that arose at the bacterial stage, after transfection for viral rescue,

or whose provenance we have not uncovered. The precautions and procedural changes we have adopted to circumvent these problems, chiefly repression of transgene expression to reduce any growth disadvantage associated with transgene over-expression, have the potential to permit derivation and large-scale amplification of viruses carrying diverse transgenes with high fidelity, and to support the continued development of adenovirus vectors as medicinal products.

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Figure 1.

Serial passage selected for ChAd63-CSN with the transgene interrupted by a bacterial transposon. **A:** Diagram of the expected structure (*top*) of the E1 region of ChAd63-CSN, showing viral inverted terminal repeat (ITR), “long” CMV promoter, CSN open reading frame (ORF), BGH polyadenylation (polyA) signal, and viral protein IX ORF (pIX); and (*bottom*) the IS150 bacterial insertion sequence disrupting the CSN ORF. Small white arrows in IS150 indicate the transposon ORFs. **B:** Discovery of the IS150 insertion by restriction mapping with *Bsr*GI (B), *Nsi*I (N), and *Pme*I (P) following 10 serial passages in

comparison to starting virus and pre-viral plasmid. Aberrant bands are shown boxed. Marker band sizes are shown in base pairs (bp).

Figure 2.

ChAd63-CSN isolate with a deletion at the CMV promoter splice acceptor site. **A:** Diagram of transgenic expression cassette of ChAd63-CSN showing “long” CMV promoter with its enhancer, exon, and intron; CSN open reading frame; and BGH polyadenylation (polyA) signal. Box indicates 66 bp deletion in the region of the splice acceptor site at the 5′ end of the intron, found in some viral isolates. **B:** Genomic restriction digest using *BsrGI* of isolate A, with the deletion, and B, lacking the deletion, before and after 10 serial passages, in comparison to pre-viral plasmid. Arrowheads indicate the reduced size of the expected 1,870 bp band, which in the non-deleted samples co-migrates with the 1,947 bp band, resulting in a doublet in isolate A (passages 1 and 10), but not in the other samples. Figures on the left indicate expected sizes of viral digest bands and those on the right indicate marker sizes.

Figure 3.

ChAd63-CSN isolates with transgene duplication and 5' nonsense extension of CSN ORF.

A: Diagram of the duplication observed in multiple viral isolates in the E1 region of the ChAd63-CSN genome, showing viral inverted terminal repeat (ITR); “long” CMV promoter; the variant duplicated CSN ORF (vCSN) with fusion to a duplicated CMV promoter at the 5' end, resulting in a nonsense extension; the unmodified CSN ORF; the non-duplicated BGH polyadenylation (polyA) site; and the viral protein IX (pIX) ORF. Predicted restriction sites are indicated with sizes of aberrant bands shown. Compare to expected structure in Figure 1A. **B:** Genomic restriction digestions of ChAd63-CSN isolates B–D with the indicated enzymes, compared to pre-viral plasmid. Figures in the middle indicate marker band sizes; those on the left the predicted viral *BsrGI* fragments; and those on the right the predicted viral *SnaBI* fragments (predicted viral *NsiI* fragment sizes are not shown). White arrowheads indicate aberrant 744 bp (*BsrGI*) and 2,614 bp (*NsiI* and *SnaBI*) fragments resulting from the duplication shown in (A). Note that the absence of the 493 bp *BsrGI* and 890 bp *SnaBI* fragments from the plasmid is expected as a result of its circularity (unlike the linear viral genome).

Figure 4. Backbone of pre-viral plasmid inserted into adenoviral transgene. **A:** Diagram of the E1 region of ChAd63-Pfs230 (*top*) showing viral inverted terminal repeat (ITR), “long” CMV promoter, Pfs230 open reading frame (tPA-Pfs230 Region C), BGH polyA signal, and viral protein IX (pIX) open reading frame; and (*bottom*), a variant identified after rescue containing the pre-viral plasmid backbone (pUC) inserted into the transgene cassette with deletion of part of the promoter and most of the open reading frame, leaving only a fragment thereof (Frag). Small white arrow in pUC represents β -lactamase (AmpR) gene. **B:** Discovery of the variant shown in (A) by PCR analysis using primers in the CMV enhancer and the BGH polyA. NC, negative control; PP, pre-viral plasmid; V1, virus lysate after rescue; V2, virus after amplification and purification. Expected size = 3,428 bp; variant size = 2,777 bp, indicated by white arrowhead. **C:** Quantitative PCR assay for β -lactamase gene (AmpR) compared to CMV promoter, expressed as copy number per 5 μ L for two limiting-dilution purified isolates (A and B) of ChAd63-CSN before (p0) or after (p10) 10 serial passages in HEK293 cells, compared to pre-viral plasmid (PP).

Figure 5.

Repression of CSN expression improves growth of ChAd63-CSN. **A:** Diagrammatic representation of the E1 region of ChAd63-CSN genome showing modified transgenic expression cassettes incorporating TetO sequences immediately downstream of the TATA boxes of either the “long” (wild-type) version of the CMV promoter or the “short” version lacking any intron or splice-sites. Also shown are the viral inverted terminal repeat (ITR), the CSN open reading frame, the BGH polyadenylation signal (polyA), and the viral protein IX (pIX) open reading frame. **B:** Single-step growth of ChAd63-CSN with either “short” or “long” TetO-containing CMV promoter in HEK293 cells or HEK293 cells constitutively expressing the tetracycline repressor (TetR) at two multiplicities of infection (MoI). **C:** Phase contrast micrographs of cell monolayers showing onset of characteristic adenoviral cytopathic effect at 48 h in TetR-expressing HEK293 cells, but not in unmodified cells.

Figure 6.

Immunogenicity, recombinant protein expression, and genetic stability of a redesigned ChAd63-CSN. **A:** Immunogenicity of CSN in Balb/c mice measured by ELISpot assay of splenocytes 14 days after vaccination with ChAd63-CSN containing either the “long” or “short” CMV promoter. Dots represent values for individual mice with line at mean and error bars showing standard error of the mean. SFC, spot-forming cells. **B:** Flow cytometry analysis of mean fluorescence intensity of HEK293 cells or TetR-HEK293 cells infected with ChAd63-CSN containing either the “long” or “short” CMV promoter and labeled with

anti-CS monoclonal antibody. Error bars indicate standard error of the mean of three replicates. **C**: Quantification of CSN protein by capture ELISA in lysates of A549 cells transduced with ChAd63-CSN containing either the “long” or “short” CMV promoter. Error bars indicate standard error of the mean of two replicates. **D**: Genomic restriction digestions using indicated enzymes of two isolates of ChAd63-CSN (“short”) before and after 10 passages in either HEK293 or TetR-HEK293 cells, compared to pre-viral plasmid.