

**High-level expression from two independent expression cassettes in replication-  
incompetent adenovirus type 35 vector**

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Replication-incompetent adenovirus type 35 (rAd35) represents a potent vaccine carrier that elicits strong, antigen-specific T- and B-cell responses in diverse preclinical models. Moreover, Ad35 is rare in human populations, resulting in the absence of neutralizing antibodies against this carrier, in contrast to the commonly used rAd5. Therefore, rAd35 is being investigated as a vaccine carrier for a number of diseases for which an effective vaccine is needed, including malaria, AIDS and tuberculosis. However, it can be perceived that effective immunization will require insertion of multiple antigens into adenoviral vectors. We therefore wanted to create rAd35 vectors carrying double expression cassettes, to expand within one vector the number of insertion sites for foreign DNA encoding antigenic proteins. We show that it is possible to generate rAd35 vectors carrying two cytomegalovirus promoter-driven expression cassettes, provided that the polyadenylation signals in each expression cassette are not identical. We demonstrate excellent rAd35 vector stability and show that expression of a transgene is not influenced by the presence of a second expression cassette. Moreover, by using two model vaccine antigens, i.e. the human immunodeficiency virus-derived Env-gp120 protein and the *Plasmodium falciparum*-derived circumsporozoite protein, we demonstrate that potent T- and B-cell responses are induced to both antigens expressed from a single vector. Such rAd35 vectors thus expand the utility of rAd35 vaccine carriers for the development of vaccines against, for example, malaria, AIDS and tuberculosis.

## INTRODUCTION

Preclinical studies have shown that replication-incompetent adenovirus type 5 (rAd5)-mediated delivery of DNA encoding immunogenic proteins of, for example, human immunodeficiency virus type 1 (HIV-1), *Mycobacterium tuberculosis* or *Plasmodium falciparum* results in a profound immune response mediated by both the cellular and humoral arms of the immune system. However, owing to the high seroprevalence and titre of neutralizing antibodies (NAb) against Ad5 in human populations, in particular in Africa, vaccine carriers derived from rare human serotypes are being investigated. One of the most advanced adenoviral vectors with low seroprevalence and low NAb titres is the human Ad35 vector, which is currently being advanced into clinical trials for malaria, AIDS and tuberculosis. However, evidence is accumulating that, in order to control HIV, the *P. falciparum* parasite or the *M. tuberculosis* bacterium effectively, a vaccine should induce immune responses against a number of proteins derived from the pathogen. In order to accommodate several DNA molecules into adenoviral vectors, a number of strategies are being actively pursued. For instance, different immunogenic proteins have been combined as fusion proteins, avoiding the need for extra regulatory sequences. Examples of these include the HIV Gag and Pol proteins (Catanzaro *et al.*, 2006; Huang *et al.*, 2001) and *M. tuberculosis* Ag85B and ESAT-6 proteins (Bennekov *et al.*, 2006; Havenga *et al.*, 2006). However, conformational changes in fusion proteins may preclude correct induction of B-cell responses or lead to undesired skewing or antigen dominance of T-cell responses. Alternative methods to co-express multiple proteins have also been described whereby, in some cases, the E1 inserts were simply extended by addition of extra expression cassettes (Belousova *et al.*, 2006; Berenjian & Akusjarvi, 2006; Gonzalez-Nicolini & Fussenegger, 2005; He *et al.*, 1998; Lee *et al.*, 2005) or by use of an internal ribosomal entry site (IRES) (Putzer *et al.*, 1997). Finally, use has been made of the knowledge in basic adenoviral biology regarding the function of the Ad-E3 region, which can be taken out of the adenoviral backbone without compromising vector production. This has led to insertion of a second expression cassette in the E3 region using either the endogenous adenoviral E3 promoter (Bauzon *et al.*, 2003; Nanda *et al.*, 2001) or a heterologous promoter (Bett *et al.*, 1994; Bramson *et al.*, 1996; Danthinne & Werth, 2000; Ghosh-Choudhury *et al.*, 1986). All strategies to expand the number of DNA molecules that can be located in an adenoviral vector have been performed by using the commonly used rAd5 vector. In this report, we describe investigations into the different ways to express inserts from multiple cassettes in a replication-deficient Ad35 (rAd35) vector. Our studies show that stable Ad35-based vectors can be obtained that are capable of expressing two different inserts with expression levels as high as those produced from single-insert vectors, using both *in vitro* studies with marker genes and *in vivo* vaccination of mice with model antigens. We therefore conclude that such rAd35 vectors expand the utility of these vaccine carriers for the development of vaccines against, for example, malaria, AIDS and tuberculosis.

## METHODS

### Plasmids, virus generation and cell culture.

PER.C6 cells (Fallaux *et al.*, 1998) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS; Invitrogen) supplemented with 10 mM MgCl<sub>2</sub>. A549 cells were maintained in DMEM with 10 % FBS. To construct viruses with a transgene in the E3 region under the influence of the E3 promoter (Fig. 1a), construct pBr.Ad35PRNΔE3.5Orf6 (Havenga *et al.*, 2006) was modified in the E3 region. pBr.Ad35PRNΔE3.5Orf6 has a deletion in the E3 region from nt 27648 to 30320, just 5' of the *MluI* site that is unique in this construct. A linker sequence corresponding to the 51 nt preceding the last open reading frame (ORF) of the E3 region (nt 30163–30214 in GenBank accession no. AY271307), followed by a *HindIII* and an *XbaI* site and flanked by *MluI* sites, was cloned in the pCR-TOPOblunt-4 vector (Invitrogen). The luciferase coding region was then introduced into the *HindIII* and *XbaI* sites and the resulting construct was cloned into pBr.Ad35PRNΔE3.5Orf6 by using the *MluI* sites. For a second construct, the luciferase gene was placed at the 5' end of the E3 region, replacing the first ORF and the polyadenylation (pA) signal of late region 4 (L4). For this purpose, a PCR fragment was generated with primers 5'-GACTAATGCAGGTGCGCAGT-3' and 5'-GTGTACGCGTCAATCGTAGCCGTCCACTG-3', amplifying the Ad35 pVIII sequences upstream of the first E3 ORF. The PCR fragment introduces an *MluI* site at the 3' end that enables cloning into pBr.Ad35PRNΔE3.5Orf6 via an internal *StuI* site at the 5' site and *MluI*. The luciferase gene was then introduced as a PCR fragment with flanking *MluI* sites by using pAdAptBsu.Luc, also used to generate viruses with luciferase in the E1 region (Havenga *et al.*, 2006), as a template. For the construction of viruses with cytomegalovirus (CMV) promoter-driven expression cassettes in the E3 region (Figs 2, 3), firstly, the different cassettes CMV-MCS-SV40pA or CMV-MCS-BGHpA were assembled in the pCR-TOPOblunt-4 vector flanked by *BssHII* sites. The CMV promoters are identical to those used for the transgenes in the E1 region and derived from pAdApt35Bsu (Havenga *et al.*, 2006). Following introduction of the appropriate transgenes (eGFP, Luc, KB9 Env-gp120 and CS) in the multiple cloning site (MCS), the entire cassettes were then transferred to pBr.Ad35PRNΔE3.5Orf6 by using the *BssHII* site flanking the expression cassettes and the *MluI* site in the receiving construct. KB9 Env-gp120 is described elsewhere (Karlsson *et al.*, 1997). The *P. falciparum* circumsporozoite (CS) gene is a synthetic insert encoding a CS protein truncated for the C-terminal 14 aa (protein sequence, GenBank/EMBL accession no. CAH04007). The N-terminal sequence of this CS protein is a consensus assembled by alignment of various sequences present in GenBank, whilst the C terminus is based upon the 3D7 *P. falciparum* clone sequence.

All viruses were generated in PER.C6 cells by co-transfection of three plasmids and produced as described previously (Havenga *et al.*, 2006).

## **Analysis of virus quality and expression.**

To investigate the stability of the expression cassettes, the different viruses were passaged serially eight times in PER.C6 cells. Following generation as described above, each virus was plaque-purified and five plaques were used for the passages. To this end,  $1.5 \times 10^6$  PER.C6 cells were seeded in six-well plates and, the next day, were infected with 5  $\mu$ l virus stock. One day after full cytopathic effect, the cells and supernatant were harvested and virus was released from the cells by two freeze–thaw cycles. Following centrifugation for 10 min at 500 **g**, the supernatant was used for the next inoculation of fresh PER.C6 cells. Mean titres ( $\pm$ SD) in these crude lysate virus stocks were  $3.6 \times 10^{10}$  ( $\pm 2.45 \times 10^{10}$ ) virus particles (VP)  $\text{ml}^{-1}$ , with mean  $\pm$ SD VP to infectious unit (VP/IU) ratios of  $13 \pm 7.47$ . At passages 2, 5 and 8, stocks were used to isolate DNA for analysis of the integrity of the expression cassettes by PCR. For this purpose, DNA was isolated by using a GeneClean Spin kit (Bio 101, Inc.) following instructions provided by the manufacturer after initial incubation of the viruses with 0.67 mg DNase I  $\text{ml}^{-1}$  at 37 °C for 30 min and further incubation with 0.2 mg proteinase K  $\text{ml}^{-1}$  in the presence of 0.4 % SDS for 1 h at 50 °C. PCR amplifications of the E1 region were done by using a forward primer hybridizing to the 5' end of the CMV promoter (5'-GTAGGTGTCAGCCTAGGTGGTC-3') and a reverse primer hybridizing to the pIX coding sequence (5'-GATGGGAGACGCCCTGTCAGATAAGG-3'). The E3 region was amplified by using primers #210 (5'-GTTTCATCTACTTCGAACTCC-3') and #195 (5'-GTAGCGGTGATCTCGTAGG-3'). Luciferase and green fluorescent protein (GFP) transgene expression was analysed by infection of A549 or PER.C6 cells seeded the day before at a density of  $5 \times 10^4$  cells per well in 24-well plates with different amounts of Ad35-based viruses. Viruses were harvested 24 h later and analysed by fluorescence-activated cell sorting (FACS) (GFP), or analysed for luciferase expression in cell lysates by using a luciferase assay system (Promega) according to the manufacturer's instructions. To analyse expression of Env and CS proteins, A549 cells were seeded at  $2.5 \times 10^5$  cells per well in a six-well plate and, the next day, infected with 1500 VP per cell. The next day, cells were washed twice with PBS and lysed in lysis buffer [20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 0.5 % deoxycholate, 1 % Tween 20, 0.5 % SDS and one proteinase inhibitor tablet in 10 ml (Roche)]. Samples were analysed by Western blotting using 1 : 5000-diluted human anti-gp120 (Polymun Scientific) with, as secondary antibody, 1 : 10 000-diluted horseradish peroxidase (HRP)-conjugated donkey anti-human IgG (Jackson ImmunoResearch) for Env protein, and 1 : 30 000-diluted rabbit anti-*P. falciparum* CS (ATCC; MRA-24, Malaria Research and Reference Reagents Resource Center) with, as secondary antibody, 1 : 30 000-diluted HRP-conjugated goat anti-rabbit (Southern Biotech Associates).

## **Animal experiments.**

All animal experiments were approved by the Institutional Review Board and the National Ethical Committee for animal experiments. BALB/c mice, 6–8 weeks old, were obtained from Harlan and were vaccinated intramuscularly (i.m.) with  $10^9$  or  $10^{10}$  VP (five to eight mice per group). Two weeks post-immunization, mice were sacrificed to isolate sera and spleens.

Spleens were used to prepare a single-cell suspension as described previously (Havenga *et al.*, 2006). Transgene-specific T-cell responses were detected by using a gamma interferon (IFN- $\gamma$ ) enzyme-linked immunospot (ELISPOT) assay. Briefly, 96-well plates (Millipore) were coated with 10  $\mu\text{g}$  anti-mouse IFN- $\gamma$  antibody  $\text{ml}^{-1}$  (BD PharMingen) and blocked with Dulbecco's PBS (DPBS; Invitrogen) supplemented with 5 % FBS. Spleen cells ( $2 \times 10^5$  and  $5 \times 10^5$  cells per well) were incubated with 100 ng peptide per well overnight at 37 °C, 10 %  $\text{CO}_2$  in culture medium. Total pools of overlapping peptides were used as immunostimulants for KB9 simian–human immunodeficiency virus (SHIV) Env (SHIV 89.6P Env Peptides Complete Set; NIH AIDS Reagent Program)- and CS (Isogen Life Science)-responsive T cells. Subsequently, the plates were washed with DPBS/0.25 % Tween 20, incubated for 3 min with distilled water (Invitrogen) to lyse any remaining cells and incubated for 1.5 h at room temperature with 100  $\mu\text{l}$  DPBS/5 % FBS containing 2.5  $\mu\text{g}$  biotin-conjugated anti-mouse IFN- $\gamma$  antibody  $\text{ml}^{-1}$  (BD Pharmingen). Next, plates were washed and incubated with DPBS/5 % FBS containing alkaline phosphatase-conjugated streptavidin (Southern Biotechnology) and incubated for 1.5 h at room temperature. Finally, plates were washed and incubated with substrate (5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium; Pierce). Spots were quantified on an ELISPOT reader (AELVIS GmbH).

Statistical analysis was performed by using SPSS software version 13.0. Data were analysed in a parametric or non-parametric way, where applicable. A log transformation was performed on the data to obtain a normal distribution, if possible. Subsequently, an ANOVA test (or Kruskal–Wallis test) was performed to test for significant differences between the groups within each experiment. If a significant difference between the groups was found, Student's *t*-test (or a Wilcoxon rank-sum test) was performed to compare the difference between each of the groups immunized with Env- or CS-containing viruses and the group injected with Ad35Bsu.empty. Post-hoc testing was performed with a correction according to Bonferroni. The difference was considered statistically significant when  $P < 0.05$ .

## RESULTS

### Identifying the need for a second heterologous promoter in the rAd35 vector

To investigate whether the Ad35-E3 promoter is strong enough to yield high-level expression of a foreign transgene located in E3, two different Ad35 vectors were generated. In the first vector, all DNA encoding ORFs 18.5, 20.3, 20.6, 10.3 and 15.2 [numbering as described by Basler & Horwitz (1996)] was removed, thereby retaining only the complete ORF 12.2, the 5' 180 bp of ORF 15.0 and the entire ORF 15.3 (Fig. 1a). Subsequently, the ORF 15.3 sequences were replaced by the luciferase reporter gene, thereby preserving the optimal splice-acceptor sequence. This strategy was chosen because a previous report indicated that mRNA levels of this E3 ORF are amongst the highest in wild-type Ad35 virus (Basler &

Horwitz, 1996). Alternatively, in vector 2, all ORFs were removed and the luciferase reporter gene was cloned directly downstream of the E3 promoter, replacing ORF 12.2. In both constructs, the natural E3 pA signal at the 3' end of the E3 region was retained to terminate transcription. In contrast to vector 2, the pA signal of L4 (pVIII) was retained in vector 1, as removal of this transcription stop could potentially result in aberrant pVIII mRNA and thus loss of vector stability, given the role of pVIII in capsid integrity (Vellinga *et al.*, 2005). Deletion of the L4 pA, however, did not compromise vector stability, as the VP/IU ratio was no different between vectors 1 and 2 in non-purified vector batches (data not shown). To determine E3 promoter strength in an optimal biological setting, i.e. in the presence of E1 proteins to *trans*-activate the E3 promoter, PER.C6 cells were infected with vector 1 demonstrating high-level luciferase activity. The luciferase activity obtained was comparable to the expression obtained from an rAd35 vector carrying the luciferase reporter gene under the control of the CMV promoter in the former E1 region (Fig. 1b). Thus, the E3 promoter is fully active in an optimal biological setting. Finally, both vectors were tested on human A549 cells, which do not express the E1 *trans*-activating proteins, demonstrating a 1–2 log lower luciferase activity than a vector carrying the luciferase reporter gene under the control of a heterologous CMV promoter (Fig. 1c). These experiments demonstrate that, although expressing foreign genes via the natural E3 promoter in an rAd35 vector is feasible and a favourable method to replicate E1<sup>+</sup> viruses, in E1-deleted viruses the level of expression is significantly lower than that obtained from a strong heterologous promoter, such as CMV. These data prompted us to design an rAd35 vector carrying two independent expression cassettes.

### **Generation of stable rAd35 vectors carrying double expression cassettes**

To investigate genome stability of an rAd35 vector carrying two identical expression cassettes, a set of rAd35 vectors was generated that carried one or two reporter genes and two identical expression cassettes in the E1 region and the E3 region (CMV-MCS-SV40pA; Fig. 2a). Subsequently, this vector was plaque-purified and five individual plaques were propagated on PER.C6 cells for eight consecutive rounds; at passages 2, 5 and 8, DNA was extracted and analysed by PCR for integrity of the transgene region. To this end, PCR was performed with primer sets that recognize Ad35 genome sequences that flank the expression cassette in either E1 or E3. In this assay, DNA bands smaller than control bands (PCR on plasmid sequences) represent deleted transgene sequences and thus are indicative for vector genome instability.

As shown in Fig. 2(a), all five plaques generated with viruses carrying Luc or GFP in the E3 region and an 'empty' cassette (promoter-pA, no transgene) in the E1 region, or with a virus carrying GFP in the E1 and Luc in E3 region, showed smaller PCR fragments after eight rounds of vector propagation in the E3-located cassettes, some of which were already visible in passage 2 and 5 samples. This demonstrates that an rAd35 vector carrying two identical expression cassettes suffers from genome instability. The deleted PCR fragments (indicated by asterisks in Fig. 2(a)) were isolated and sequenced to investigate the nature of the

deletions (data not shown), confirming that the fragments were virus-derived and included deletions of (part of) the expression cassette.

We next attempted to negate the homology by generating rAd35 vectors in which one of the SV40pA signals was replaced by a bovine growth hormone (BGH) pA signal. For this purpose, again a set of viruses was generated that contained one or two transgenes (Fig. 2b). The viruses were analysed for genome stability after eight passages of five plaques, as described above. rAd35 vectors with luciferase in the E3 cassette and an empty cassette or eGFP in E1 showed four of five stable virus stocks, irrespective of the location of the heterologous BGHpA. The samples taken at passages 2 and 5 showed complete stability in all cases. If the E3 region contained a GFP cassette, all virus stocks showed the expected band and no deletions were observed (Fig. 2b). This demonstrates that it is possible to incorporate two CMV-driven expression cassettes stably in an rAd35 vector, provided that the pA signals are heterologous.

To test expression levels derived from the expression cassette located in either the former E1 region or E3 region, rAd35 vectors carrying the luciferase or GFP reporter genes or both reporter genes simultaneously were produced and purified. To investigate the possibility of promoter interference, rAd35 vectors carrying only one expression cassette in the former E1 region were included as controls (Fig. 3a). The vectors were used to transduce human A549 cells and levels of GFP or luciferase were assessed. As shown in Fig. 3(b), expression of luciferase or GFP obtained from any vector was comparable with that obtained from all other vectors. A 2- to 3-fold difference in expression level was only seen for luciferase expression from expression cassettes in the former E1 or E3 regions (Fig. 3b). This difference was not observed when eGFP was used as transgene (Fig. 3c). These results demonstrate that high-level expression of reporter genes in an rAd35 vector could be obtained, irrespective of the location of the expression cassette (E1 or E3) and irrespective of the number of expression cassettes present in one vector (one versus two cassettes).

### **Immune responses against antigens cloned into an rAd35 vector carrying two expression cassettes**

To assess the influence of multiple expression cassettes and their location in rAd35 vectors in a more relevant biological setting, a panel of rAd35 vectors carrying the coding domain for the SHIV (KB9)-derived Env or the *P. falciparum*-derived CS antigen was generated and purified. Comparison of immune responses obtained from a vector carrying Env and CS versus Env alone (Fig. 4a, vectors 1 and 2) allows investigations into promoter interference. Likewise, head-to-head comparison of a vector carrying the CS protein in either the E1 or E3 expression cassette allows investigations into possible differences due to expression-cassette positioning in the rAd35 backbone (Fig. 4a, vectors 3 and 4). All four vectors could be produced without any indication of genome instability, as no DNA fragments smaller than the plasmid controls could be detected in either the E1 or E3 expression cassette (Fig. 4b). As indicated in Fig. 4(c), proper expression of both the Env and CS proteins was obtained

from all vectors constructed. Immunization of BALB/c mice with an rAd35 vector carrying the CS coding sequence in an E1-located expression cassette did not result in significant differences in either anti-CS T-cell responses (Fig. 5a) or B-cell responses (Fig. 5b), compared with a vector carrying the CS coding domain in an expression cassette in the former E3 region of an rAd35 vector. These results thus demonstrate that the CS protein level derived from a CMV-SV40pA expression cassette located in E1 is comparable with the CS protein level derived from the CMV-BGHpA cassette located in the former E3 region of the rAd35 vector.

Next, a vector carrying both Env and CS (vector 1; see Fig. 4a) was compared directly with a mixture of two vectors, one carrying Env only and one carrying CS only [vectors 2 and 4; see Fig. 4(a)], and compared further with a one-vector immunization, whereby the vector carried only one antigen (vector 2 in case of Env read-out or vector 4 in case of CS read-out). The data obtained demonstrate that anti-Env T-cell responses are comparable in each setting (Fig. 6a), indicating that (i) antigenicity of Env is not affected by the presence of a second vector in the vaccine mixture, in this case expressing CS protein, and (ii) antigenicity of Env is not affected by the presence of a secondary expression cassette encoding the CS antigen in a single rAd35 vector. However, anti-CS T-cell responses obtained from an rAd35 vector carrying both Env and CS were significantly lower than anti-CS T-cell responses obtained with a single vector carrying CS alone or a mixture of two vectors, one carrying Env, the other CS (Fig. 6b). This difference cannot be explained by differences in expression level of the CS protein, given the results described in Fig. 5 and the fact that no differences could be observed in anti-CS B-cell responses between the three vaccines (Fig. 6c). Therefore, the difference may suggest antigenic dominance of Env over CS when expressed in the same antigen-presenting cell. Finally, we assessed whether higher dosing could overcome the lower anti-CS T-cell response and therefore a 10-fold higher dose ( $10^{10}$  VP) was used for mice immunization. The data obtained demonstrate that, at  $10^{10}$  VP, no statistically significant differences are observed in anti-CS T-cell responses when vaccinating with a mix of two vectors versus a single vector carrying both antigens simultaneously (Fig. 7a), whereby Env responses were identical at both  $10^9$  and  $10^{10}$  VP doses (Fig. 7b). Thus, dependent on the antigens selected, antigenic dominance can occur in a single vector carrying two antigens, but higher dosing can apparently be used to correct this phenomenon.

## DISCUSSION

The data presented here demonstrate that the technology developed permits the use of rAd35 vectors carrying double expression cassettes, in order to expand the number of foreign genes that can be inserted separately into these vaccine carriers. To our knowledge, this is the first study investigating strategies to express multiple transgenes from a single non-rAd5 vector and the results obtained are probably directly transferable to other rare

human or non-human adenovirus serotypes under development as vaccine carriers. As shown, we initially attempted to express the second insert from an internal E3 promoter, thereby investigating two options: (i) making use of the splice-acceptor sequence of the 15.3 kb mRNA, based on the expected high level of expression of this mRNA (Basler & Horwitz, 1996), and (ii) inserting the transgene behind the E3 promoter, thereby replacing all ORFs in the native Ad35 E3 region. Although in both cases, expression of the marker gene was readily detectable in the presence of E1 proteins, an unacceptable reduction in expression level of the second transgene was observed in the absence of E1 proteins. This phenomenon has been shown by ourselves and others (Nanda *et al.*, 2001) when using rAd5, demonstrating higher expression (by over three orders of magnitude) from the E3 promoter when E1A was present. Thus, strategies pursuing a replication-competent Ad35 vector, i.e. carrying E1, could make use of the endogenous rAd35 E3 promoter for expression of a secondary gene. However, for replication-deficient Ad35 vectors, we next investigated rAd35 vectors carrying two foreign expression cassettes. As the CMV promoter is known to be one of the strongest promoters in mammalian cells, we first investigated stability of an rAd35 vector carrying two identical, CMV promoter-containing expression cassettes. Alternative solutions to insert two CMV cassettes in E1 or to use an IRES sequence were not pursued, due to expected instability problems during the adenovirus replication cycle and lower expression levels of the second transgene, respectively. The Ad35 vector with two identical CMV promoter-driven cassettes in the E1 and E3 region was first tested for genome stability, as described previously (Havenga *et al.*, 2006), by performing eight serial passages on E1-complementing cells (PER.C6 cells). Subsequent PCR analysis of the expression cassettes was performed to identify recombination events. As observed, insertion of two identical expression cassettes into rAd35 clearly compromised genome stability, as witnessed by multiple PCR products that were smaller than the expected PCR product, resulting in the disqualification of this configuration of an rAd35 vector carrying double expression cassettes. Sequence analysis revealed that one of these fragments may have been caused by homologous recombination with the cassette located in the E1 region. This deletion type was already present in the passage 2 and 5 samples. Recently, homologous recombination was also observed in an Ad5-based vector carrying two identical expression cassettes head to tail and located in the E1 region (Belousova *et al.*, 2006). In that study, the rAd5 instability was countered by changing the secondary expression cassette (both promoter and pA signal) completely. However, the choice of the second promoter in this study (SV40 promoter) lowers the expression level of the secondary gene considerably, which was apparently still acceptable for the intended use of that particular vector. The fact that the expression cassettes in the rAd35 vector were not both located in the E1 region is not considered to be an advantage over the alternative strategy, because recombination is considered to occur between two different adenovirus genomes during the replication cycles, as was hypothesized and visualized in previous studies involving homologous recombination in adenoviral vectors (Murakami *et al.*, 2002; Steinwaerder *et al.*, 1999). The smaller PCR fragment seen in all virus preparations with

homologous expression cassettes (Fig. 2a) does not seem to be the result of a homologous recombination event with the expression cassette in the E1 region because, in that case, the entire cassette was deleted. The nature of this deletion is unclear, but it is not caused by specific SV40pA sequences in the E3 region, as viruses with BGHpA in E1 and SV40pA in E3 are stable (Fig. 2b).

As shown, we next assessed genome stability of an rAd35 vector in which the pA signal was not identical. We chose the BGHpA to substitute the SV40pA sequence because we observed no differences in mRNA stability or protein expression level using either of the two pA signals, at least in the context of plasmid DNA (data not shown). The use of such a configuration gave rise to an rAd35 vector that maintained genome stability for eight passages in four of five tested cases. Eight passages are considered necessary for large-scale production campaigns to produce millions of vaccine dosages. The subsequent expression data obtained demonstrated highly similar expression levels derived from luciferase or GFP, irrespective of the location of these genes in the E1 or E3 region. Most convincingly, the B- and T-cell responses obtained in mice immunized with a vector carrying the *P. falciparum*-derived CS protein in either the E1 region or the E3 region were indistinguishable, demonstrating that infection, expression and processing of an antigen expressed from the E3 region are identical to those of an antigen expressed from the E1 region. Finally we tested an rAd35 vector carrying one antigen from HIV and one from *P. falciparum* and compared this vector with control vectors carrying only one antigen in the respective adenoviral region. After confirmation of genome stability of the manufactured vaccine and mouse immunization, we observed a loss in anti-CS T-cell induction in the vector carrying the CS gene in the E3 region without compromising anti-CS antibody responses, compared with a vaccine in which the two vectors were mixed. Given the observation that the T-cell response to the HIV-derived Env protein was equal between the single-vector and double-vector vaccines, we concluded that the lower T-cell response against CS could not be due to differences in the quality of the vector. Also, given the observation that the anti-CS antibody response was comparable between the single vector and double vector, we concluded that expression of the CS antigen was not in any way compromised by the location of the CS cDNA in the adenoviral backbone. One plausible reason for the reduced anti-CS T-cell response is that the presence of the Env-gp120 protein within the same antigen-presenting cell is dominant and, thereby, at least partly prevents an adequate response to the CS protein. This phenomenon is well known for CD8 T-cell epitopes and occurs not only following co-expression of two or more immunogenic peptides or proteins, but also between different epitopes within one protein (reviewed by Yewdell & Bennink, 1999). In general, immune dominance occurs as a result of specificities of proteolytic cleavage, selectiveness of peptide transport and competition for major histocompatibility complex class I binding, as well as diversity of T-cell receptor recognition. In a recent study where HIV-1 Env was combined with HIV-1 p55-Gag (Toapanta *et al.*, 2007), a significant reduction was observed in the anti-Gag T-cell response. The authors

suggested that this interference was specific for the HIV-1 Env and Gag combination, as simian immunodeficiency virus (SIV) and equine infectious anemia virus Env proteins did not show a reduction of the Gag response and the interference was not seen when an SIV Gag protein was used instead of HIV-1 Gag. The results presented here suggest that the interference caused by HIV-1 is broader, also affecting the T-cell response to malaria parasite-derived CS proteins. Interestingly, we did not observe immune dominance when the two proteins were expressed from two different Ad35-based vectors. It has been described previously that, when dominant and subdominant epitopes are expressed separately from (plasmid) vectors injected into two different flanks of mice, the response to the subdominant epitope is restored, as if the dominant epitope were absent (Liu *et al.*, 2006; Rodriguez *et al.*, 2002). In our study, however, the different adenoviral vectors were mixed before injection, so the absence of Env dominance can only be explained by assuming that sufficient dendritic cells presented CS peptides only. In the study by Liu *et al.* (2006), immune dominance was still observed following mixing of the two DNA plasmids with dominant and subdominant epitopes. It is possible that, besides the fact that the studies involved different sets of epitopes, differences in expression levels between plasmid-based and Ad35-based immunization account for this discrepancy. In line with this theory, we demonstrate that increased dosing can restore the loss of anti-CS T-cell response.

In conclusion, the data obtained demonstrate that rAd35-based vectors expressing multiple inserts can be generated without compromising either virus genome stability or level of expression of the inserts. Within the boundaries of the Ad35 packaging capacity, this enables combinations of DNAs encoding different immunogenic proteins from one single pathogen or perhaps targeting multiple disease areas. The results obtained with the two antigens in this study also show that careful investigations as to which antigens are combined in one vector must be applied to prevent occurrence of immune dominance.

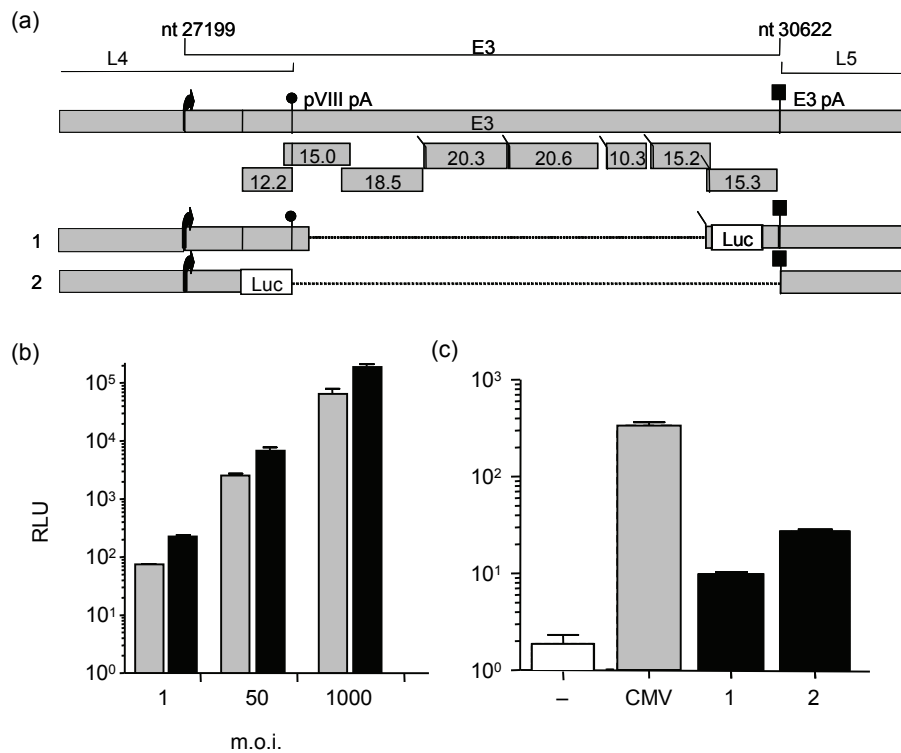
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**Fig. 1.** (a) Schematic presentation of the E3 region of Ad35 and luciferase marker-gene location. The E3 promoter is indicated by black arrows and the coding regions [as described by Basler & Horwitz (1996)] are presented by their predicted molecular masses (kDa). ● indicates the positions of the pA site of late region 4 (L4) and ■ indicates the position of the pA site of the E3 region. Diagonal lines indicate splice-acceptor sites preceding the E3 coding regions. (b) Luciferase marker-gene expression measured in relative light units (RLU) detected after infection of E1-containing PER.C6 cells with three different VP per cell ratios of construct 1 (black bars) or control vector (grey bars). As control, a vector carrying the luciferase gene driven by the CMV promoter inserted in the E1 region was used. (c) Luciferase marker-gene expression, measured in RLU, detected after infection of A549 cells with 1000 VP per cell of either construct 1 or 2 (black bars) or control vector (grey bar). As control, a vector carrying the luciferase gene driven by the CMV promoter inserted in the E1 region was used, whereas non-transduced cells (white bar) were used to set the background.





**Fig. 4.** (a) Schematic presentation of rAd35-based double-insert vectors. The E1-located expression cassettes are terminated by SV40pA sequences, whereas the E3 cassettes are terminated by the BGHpA sequence. Env, HIV-derived gp120 envelope protein; CS, *P. falciparum*-derived circumsporozoite protein; e, empty, meaning no transgene. Vectors with one insert in E1 have a deletion in the E3 region, whereas vectors with one insert in the E3 region have an expression cassette in E1 (CMV-pA). (b) Agarose gels loaded with PCR products, produced using purified vectors as template, to assess genome stability. Lane numbers correspond to virus numbers as listed in (a). Size markers are shown (kb). Plasmids P<sub>1</sub>–P<sub>4</sub> correspond to the controls used for each of the PCR amplifications. (c) Western blot analyses of protein lysates prepared from human A549 cells infected 48 h previously with 1500 VP per cell, using antibodies specific for HIV-1 Env (left) or *P. falciparum* CS (right). Lane numbers correspond to virus numbers in (a). Size markers are shown (kDa).

**Fig. 5.** Vaccine potency obtained after a single i.m. administration ( $10^9$  VP) of an rAd35 vector carrying CS DNA in the E1 region [C(E1)] or E3 region [C(E3)]. Two weeks after immunization, BALB/c mice ( $n=8$ ) were sacrificed and T cells were isolated from spleens. (a) Responses were measured by using an IFN- $\gamma$  ELISPOT to total CS peptide pool. s.f.u., Spot-forming cells. (b) Serum of vaccinated animals was analysed for the presence of CS-specific antibodies. Control administrations to determine background levels in the assays employed were performed with rAd35 vector carrying an expression cassette in the E1 region without transgene (empty).

**Fig. 6.** (a) Vaccine potency obtained after a single i.m. administration ( $10^9$  VP) of either an rAd35 vector carrying Env in the E1 region (single), a mix of two rAd35 vectors, one carrying Env in E1 and the second vector carrying the CS coding DNA in E1 (mix), or a single rAd35 carrying Env in the E1 region and CS in the E3 region (double). Two weeks after immunization, BALB/c mice ( $n=8$ ) were sacrificed and T cells were isolated from spleens. Responses were measured by using an IFN- $\gamma$  ELISPOT. No statistical differences could be observed in the anti-Env T-cell response between animals vaccinated with single, mix or double vaccines. (b) Similar to (a), with the exception that read-out was performed on CS T-cell induction using CS peptide pool and with the exception that the single vaccine consisted of an rAd35 vector carrying CS coding DNA in the E3 region. The CD8 T-cell responses to CS protein expressed by all types of vector proved to be significantly higher than those to control virus ( $10^{10}$  VP Ad35.empty). The anti-CS T-cell response to the 'mix' was found to be significantly higher than that to the double vaccine.  $*P<0.02$ . The difference between the single and double groups was not statistically significant. (c) Antibody read-out in serum derived from BALB/c mice immunized with single, mix, or double vaccine modality as described for (b). The B-cell responses (serum antibodies) to CS protein were significantly different from those to control virus, but not between CS groups.

**Fig. 7.** Groups of BALB/c mice were injected with two doses ( $10^9$  and  $10^{10}$  VP) of a mix of vectors 2 and 4 (see Fig. 4) or of the double-insert vector 1 (see Fig. 4). T-cell response to CS protein (a) and Env protein (b) was measured as described in Methods. The difference between the CS groups 'mix' and 'double' is statistically significant at  $10^9$  VP ( $P < 0.05$ ), but not significant at  $10^{10}$  VP ( $n=5$ ).