



## BRIEF COMMUNICATION

# Adenovirus-mediated expression of green fluorescent protein

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A recombinant replication-deficient adenovirus has been generated that expresses a mutant of the *Aquorea victoria* green fluorescent protein (GFP) under the control of the strong CMV promoter by insertion into the E1 region (AdV-GFP). High expression of GFP was found in different cell types after infection with the recombinant virus that could be easily detected by fluorescence microscopy. In human umbilical vein endothelial cells (HUVEC), expression levels had already reached a maximum after 2 days and were stable for at least 7 days, as determined by Western

blotting. As demonstrated by FACS analysis, up to 98% of HUVEC and approximately 70% of human smooth muscle cells could be transduced to express GFP. Since GFP can be detected in cells without the need for prior fixing and staining, the virus should be useful for optimizing in living cells the transduction efficiency of different cell types, of cells from different experimental animals, as well as studying the kinetics and persistence of adenovirus-mediated gene transfer in diverse experimental settings.

**Keywords:** green fluorescent protein; recombinant adenovirus; marker gene; endothelium

Gene transfer techniques using replication-deficient recombinant adenoviral vectors (AdV) have considerable potential both at experimental and therapeutic levels.<sup>1–3</sup> Advantages include the ability to produce high-titer stocks, the high efficiency of gene transfer into a variety of cell types, and the ability to transduce cells that have a low mitotic index, eg the endothelium, kidney and cells of the central nervous system.<sup>4,5</sup> Limitations of AdV include the relatively short time of expression of the (therapeutic) gene *in vivo* (a few weeks to several months, depending on the organ) due to the episomal maintenance of the adenoviral DNA in the cell, and the elicitation of an immune response by the host organism.<sup>6–8</sup> These features favor, in the first instance, applications where only a transient expression of the ectopic gene is desirable, eg inflammatory reactions.

Despite its broad cell-type specificity, considerable differences regarding the ability to transduce different cells by adenoviral vectors have been observed. Moreover, even larger differences exist between men and other mammalian species.<sup>9,10</sup> We have noted an approximately 10-fold lower transduction efficiency of adenovirus vectors in porcine as compared with human endothelial cells.<sup>11,12</sup> This species difference leads to difficulties in evaluating AdV gene therapy in experimental animals. Therefore, a careful optimization of the conditions used is of great importance to achieve optimal transduction efficiencies. For those purposes, several adenoviral vectors have been constructed that express marker genes such as  $\beta$ -galactosidase and luciferase that are detected by relatively simple enzymatic assays.<sup>13,14</sup> However, none

of them can be directly visualized in living cells without the need for prior incubation with substrates or staining procedures, a feature fulfilled by green fluorescent protein.

Originally isolated from the jellyfish *Aquorea victoria* as a 238 amino acid protein,<sup>15</sup> wild-type GFP emits a bright green fluorescence upon excitation at both 395 and 475 nm. Crystallization has revealed a cylinder-like structure of  $\beta$ -sheets wrapped around a single central helix.<sup>16</sup> The fluorophore results from the autocatalytic cyclization between amino acids of the polypeptide backbone and oxidation of the  $\alpha$ - $\beta$  bond of the central tyrosine. Due to this rigid encapsulation, the fluorophore is remarkably insensitive to changes in environmental conditions, eg pH, O<sub>2</sub> quenching of the excited state, and to proteolysis. The generation of mutants that show improved levels of fluorescence as well as different excitation and emission maxima has further triggered the widespread use of GFP for gene expression and protein localization studies.

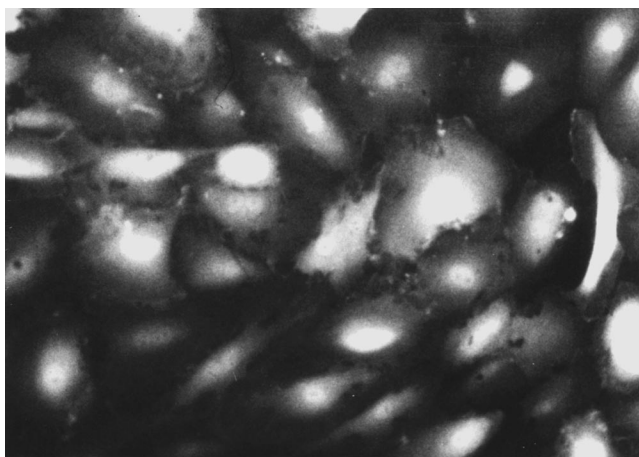
We have used the 'cycle 3' GFP mutant (C3) generated by DNA shuffling that displays a 42-fold higher fluorescence in eucaryotic cells as compared to the wild-type protein.<sup>17</sup> C3 excitation and emission maxima are at 488 and 510 nm, respectively, allowing detection by fluorescence microscopy and FACS analysis using standard filter sets. After cotransfection of the transfer vector<sup>18</sup> containing C3 with the plasmid pJM17<sup>19</sup> into 293 cells, fluorescence originating from the expression of the nonrecombined plasmid could already be detected in the transfected cells after 16 h, allowing direct determination of transfection efficiency. At later stages, after recombination had occurred and 'rounded' cells and plaques were formed, spreading of the infection could directly be followed by fluorescence. Expression was surprisingly short-lived (approximately 2 days), but allowed the

identification of even small positive plaques at an early stage of infection.

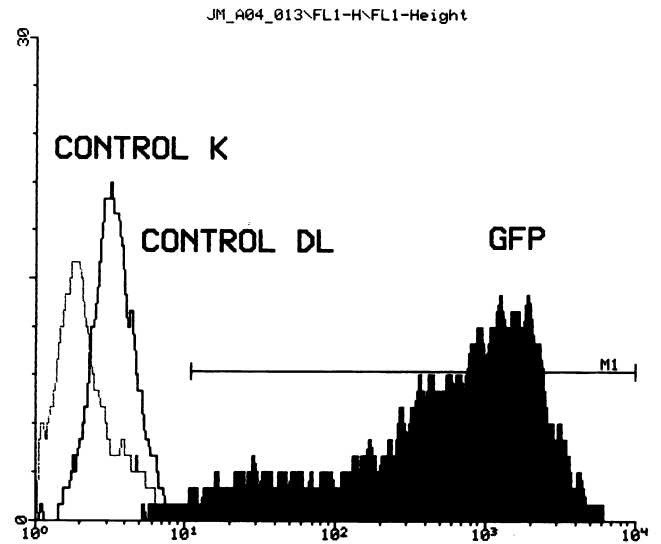
The vascular wall has become an important target for gene therapy for a variety of pathophysiological conditions including inflammation, tumor angiogenesis, transplantation and restenosis following balloon angioplasty.<sup>1,9,10,20</sup> We have therefore tested the AdV-GFP by infecting endothelial cells and smooth muscle cells. Human umbilical vein endothelial cells (HUVEC) were infected with AdV-GFP at a multiplicity of infection (MOI) of 100 and expression of GFP was visualized daily under the fluorescence microscope. Under the conditions used, virtually all cells showed a green fluorescence, as shown in Figure 1, taken at day 3 after infection. To quantify more accurately both the number of cells and the amounts of fluorescence, HUVEC were trypsinized and analyzed by FACS. As shown in Figure 2, 99.8% of the HUVEC expressed GFP, as compared with cells infected with a control virus (dl312) or non-infected cells. The main population of cells expressed GFP levels two to three orders of magnitude higher than the control cells. In contrast, when human smooth muscle cells were infected under the same conditions, only approximately 72% of the cells expressed GFP, as determined by FACS analysis (Figure 3). Also, the average intensity of fluorescence was lower as compared with HUVECs, with the majority of the cells showing approximately 10-fold higher fluorescence than the controls.

The kinetic of adenovirus-mediated GFP expression was determined by Western blotting (Figure 4). A specific band of approximately 27 kDa corresponding to the expected size of GFP was detected in lysates from AdV-GFP-infected HUVEC by an anti-GFP antibody 24 h after infection. GFP levels further increased at day 2 after infection and remained stable up to day 7, with only a minor decrease at day 10, which is probably due to loss of HUVEC after prolonged time of confluence in culture.

In summary, we have demonstrated that GFP can be expressed by recombinant adenovirus at levels that can

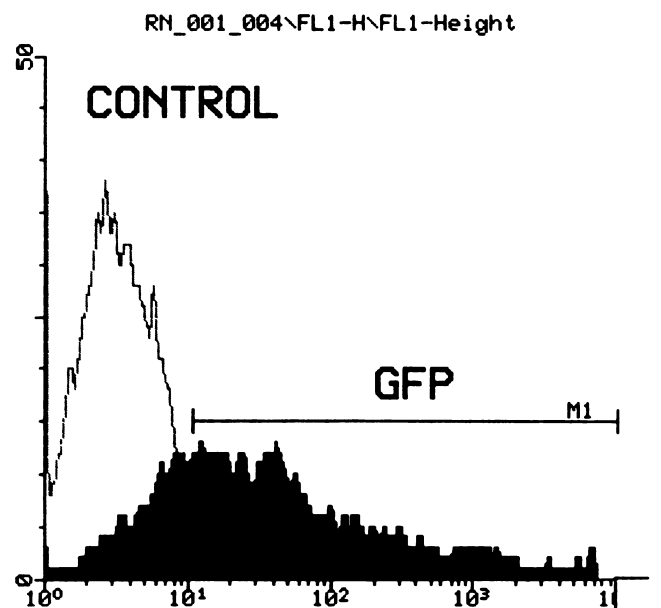


**Figure 1** Fluorescence microscopy of GFP expressing cells. A 740 bp *XbaI-HindIII* (partial) fragment from the plasmid alpha-GFP cycle 3 was cloned into the adenoviral transfer vector pACCMVpLpASR<sup>+</sup><sup>18</sup> and recombinant adenovirus obtained after cotransfection into 293 cells together with the plasmid pJM17.<sup>19</sup> HUVEC were isolated as described<sup>21</sup> and infected at an MOI of 100 for 30 min in complete PBS, then washed and grown further in fresh medium. Pictures were taken 3 days later under the fluorescence microscope using the FITC channel.

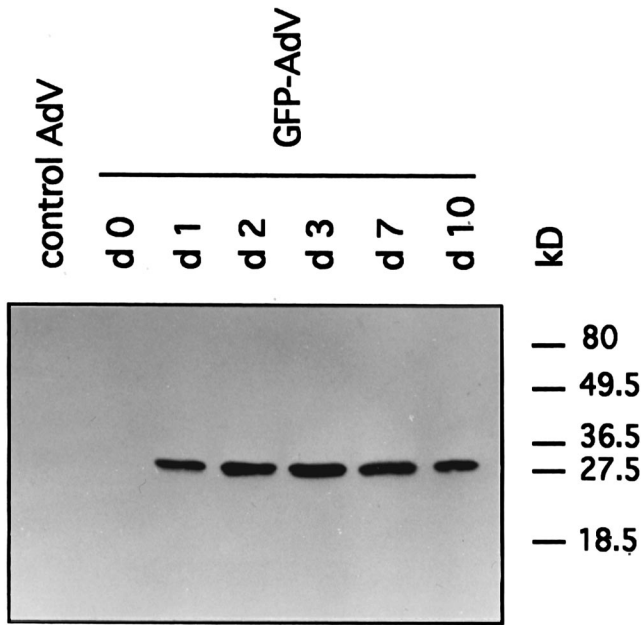


**Figure 2** FACS analysis of AdV-GFP-infected EC. HUVEC were grown to confluency and infected with either the AdV-GFP, with control virus (dl312), or left non-infected as described for Figure 1. After 3 days, cells were trypsinized and analyzed by FACS using FITC filters. Of the AdV-GFP-infected cells 99.8% were positive as compared with the virus control.

be easily detected in living cells by conventional fluorescence techniques, without the need for enzymatic assays or immunological staining. This vector can therefore serve as a valuable tool for optimizing adenovirus transduction protocols. Moreover, recombinant viruses that express GFP in addition to a therapeutic gene could be generated to follow the success of infection and distribution of the vector *in vivo*.



**Figure 3** FACS analysis of AdV-GFP-infected human smooth muscle cells. Cells were infected either with AdV-GFP, or left non-infected as described for Figure 1. After 3 days, cells were trypsinized and analyzed by FACS using FITC filters. Of the AdV-GFP-infected cells 72% were scored positive as compared with control cells.



**Figure 4** Kinetics of adenovirus-mediated GFP expression. HUVEC were grown in 24-well plates and infected with AdV-GFP as described for Figure 1. Cells were harvested into Laemmli buffer<sup>22</sup> at days 0, 1, 2, 3, 7 and 10 as indicated above each lane and analyzed by Western blotting using an anti-GFP polyclonal antibody (Clontech, Palo Alto, CA, USA); dl312, control virus; molecular weight in kDa is indicated on the right.

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