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Visualization of herpes simplex virus type 1 virions using fluorescent colors

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A B S T R A C T

Our laboratory was one of the first to engineer a live fluorescent tag, enhanced green fluorescent protein (eGFP), that marked the capsid of herpes simplex virus type 1 (HSV-1) and subsequently maturing virus as the particle made its way to the cell surface. In the present study we sought to increase the repertoire of colors available as fusion to the small capsid protein, VP26, so that they can be used alone or in conjunction with other fluorescent tags (fused to other HSV proteins) to follow the virus as it enters and replicates within the cell. We have now generated viruses expressing VP26 fusions with Cerulean, Venus, mOrange, tdTomato, mCherry, and Dronpa3 fluorescent proteins. These fusions were made in a repaired UL35 gene (VP26) background. These fusions do not affect the replication properties of the virus expressing the fusion polypeptide and the fusion tag was stably associated with intranuclear capsids and mature virions. Of note we could not isolate viruses expressing fusions with fluorescent proteins that have a tendency to dimerize.

Visualization of virus particles in cells using light microscopy was significantly advanced by the use of live fluorescent reporters such as green fluorescent protein (GFP) derived from the jellyfish (*Aequorea victoria*) (Chalfie et al., 1994). For herpes simplex virus (HSV) the ability to generate a fusion polypeptide between a virion protein and GFP allowed one to follow in living cells the virus particle as it assembles in the nucleus, exits this structure and begins on the egress pathway to the cell surface. The first two such recombinant viruses made utilized a tegument protein, VP22 (Elliott and O'Hare, 1999) and the small capsid protein, VP26 (Desai and Person, 1998) to incorporate the fluorescent protein (FP) into the virion. In addition, these and subsequent engineered fusion proteins facilitated the ability to visualize virus entry, cell to cell spread and capsid translocation on the cellular cytoskeleton (Antinone et al., 2006; Bearer et al., 2000; Bohannon et al., 2012; Conway et al., 2010; de Oliveira et al., 2008; del Rio et al., 2005; Desai et al., 2008; Donnelly and Elliott, 2001; Hutchinson et al., 2002; La Boissiere et al., 2004; Maier et al., 2016; Nagel et al., 2012; Nagel et al., 2008; Ogasawara et al., 2001; Radtke et al., 2010; Scherer et al., 2016; Smith et al., 2001; Sole et al., 2007; Sugimoto et al., 2008; Taylor et al., 2012; Toropova et al., 2011). In this paper we expand on the repertoire of fluorescent fusions available for the small capsid protein (VP26) of HSV type 1 strain KOS. This VP26 tag is one of the most useful and most used reporters for visualizing all aspects of HSV-1 replication. Although the first engineered version was a GFP fusion we have now engineered several additional colors that can be used individually or in combination for visualizing the virus in living cells.

The 5.2-kb *Eco*RI L fragment of KOS (Fig. 1) cloned into pUC19 and designated pKEL has been previously described (Desai and Person, 1998; Desai et al., 1998). This fragment spans nucleotide 69641–74872 of the KOS genome (Macdonald et al., 2012). The plasmid encodes UL35 and the C-terminal portion of UL34 and UL36 genes. When the 5′ end of the UL35 gene encoding VP26 was engineered using overlap PCR methods to introduce an XhoI restriction enzyme site for cloning purposes there was an inadvertent mutation generated that essentially deleted 65 nucleotides upstream of the UL35 ATG as well as the first 5 codons (Fig. 1). This plasmid was designated pKEX (Desai et al., 1998). Subsequently a linker sequence comprising the NcoI and BsrGI restriction enzyme sites was introduced into the XhoI site in order to clone and create a translation fusion of GFP at the N-terminus of VP26 (Desai and Person, 1998). It was assumed that the GFP open reading frame

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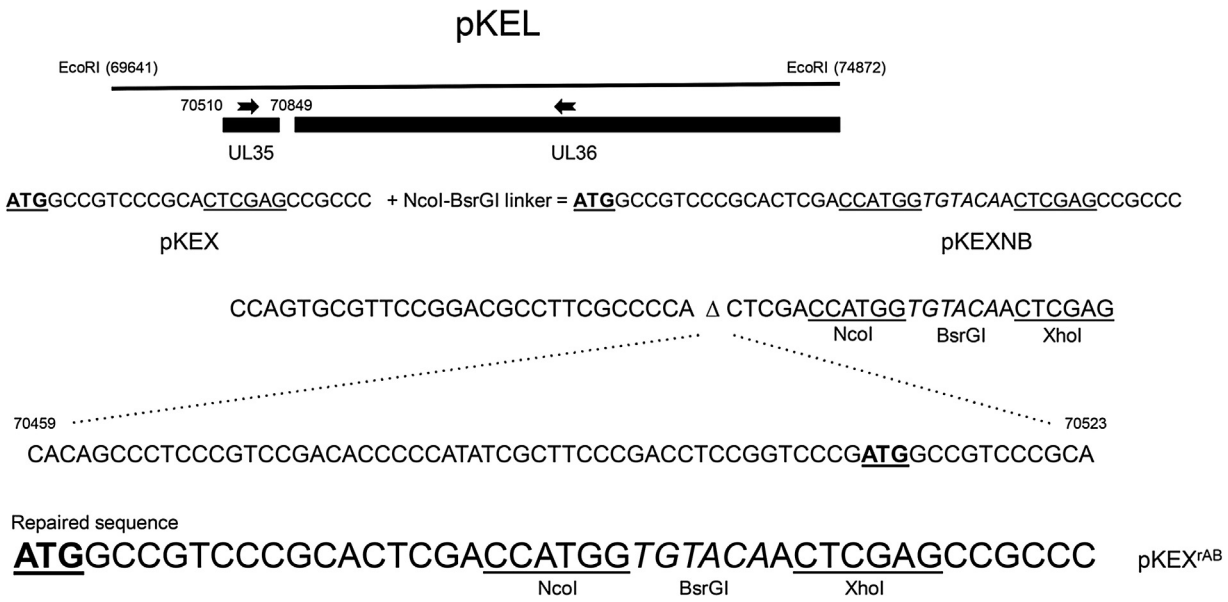

Fig. 1. Engineering of the upstream region of the UL35 gene. Shown in the figure is plasmid pKEL, which encodes the EcoRI L fragment of HSV-1 strain KOS (Macdonald et al., 2012). The UL35 gene and part of the UL36 gene are shown. Below this are shown the predicted nucleotide sequences of the design to engineer the XhoI site in pKEX and the NcoI-BsrGI sites in pKEXNB. The sequence that was deleted during this engineering and the deletion site (Δ) is shown below and spans nucleotides 70,459–70,523 of KOS. The UL35 start codon is underlined and in bold. The repaired sequence is shown below this starting with the UL35 ATG and the engineered NcoI, XhoI (underlined) and BsrGI (italics) restriction enzyme sites. ORFs encoding Cerulean and Venus genes were cloned using NcoI and BsrGI restriction enzyme sites. ORFs encoding Dronpa3, mOrange, tdTomato, mRFP and mCherry were cloned using the BsrGI and XhoI sites in pKEX^{rAB}.

(ORF) was fused to the first five codons of VP26 and at the C-terminus to the rest of VP26. The deletion only became apparent after later investigations (Baines, J and Sodeik, B., pers comm.). Fortuitously, the ATG start codon present in the NcoI restriction site enabled translation of GFP fused in frame with VP26 and the virus K26GFP (Desai and Person, 1998) was derived and used for subsequent analysis both by the community and us. This virus replicated with normal wild-type properties in cell culture.

We sought to repair this deletion and then engineer in the different fluorescent genes. PCR methods were used to correct the introduced deletion. To do this we made use of a HpaI restriction site at 70257 and a BstBI site at 70959 (Macdonald et al., 2012). Two PCR products were amplified, one (pcr A) spans the HpaI to the original XhoI site inserted in UL35 and the second (pcr B) from that same XhoI site to the BstBI site (Table 1). Plasmid pKEL-XhoI was first digested with HpaI and XhoI to clone pcr A fragment. This plasmid pKEX^{rA} was sequenced to confirm authentic sequence information and then digested with XhoI and BstBI to clone pcr B. The final plasmid after sequence verification was named pKEX^{rAB} (Fig. 1). This plasmid corrected the mutation but retained the restriction cloning sites that were engineered as part of the original cloning strategy. Thus spanning the first 5–7 codons of UL35 we inserted an NcoI-BsrGI-XhoI engineered sequence encoding restriction sites that were used to clone the different genes expressing fluorescent tags (Fig. 1). The ATG that is part of the NcoI restriction enzyme site is in-frame with that of the UL35 gene. Thus, most of the FP genes can be cloned using Nco1-BsrG1 sites, as these are the sites used to clone the first series of FP genes in the different expression vectors made by Clontech. However, many of the red variants contain an internal NcoI site and so one can use the BrGI-XhoI cloning sites. The fluorescent protein ORFs were amplified by PCR using the following plasmids as templates: pCerulean-VSVG (Cerulean) (Presley et al., 1997), pYFP-N1 (Yellow) (Clontech), pVenus-VSVG (Venus) (Presley et al., 1997), pFBCmCherry (mCherry) (Luitweiler et al., 2013), pCAG2LMKOSimO (mOrange) (Kaji et al., 2009), FUtdTW (tandem dimer tomato) (Rompani and Cepko, 2008), Dronpa3-N1 (Dronpa3) as the template using primers listed in Table 1. The PCR products were cloned into the engineered restriction sites (Fig. 1 and Table 1) in UL35 at codon 5, creating a translational fusion between these two genes. All the fluorescent gene ORFs were cloned as XhoI-BsrGI fragments, except for the Venus and Cerulean genes which were cloned as NcoI-BsrGI fragments into the respective sites in pKEX^{rAB}. The plasmid and virus encoding VP26-mRFP1 fusion was generated in a previous study but is analyzed here together with all the new fluorescent tags (Desai et al., 2008). The YFP ORF in this study was cloned into the original pKEXNB, which contains the deletion and was used to derive a virus expressing this fusion.

The different gene fusions were then recombined into the KOS virus genome using marker-transfer methods. For all transfections and infections we used Vero cells, or a transformed Vero cell line, C32 (Person and Desai, 1998) and for virion preparations we used human embryoninc lung (HEL) cells grown in minimum essential medium- alpha medium supplemented with 10% fetal calf serum (Gibco-Invitrogen) and passaged as described previously (Desai et al., 1998). C32 cells were used because they tend to give rise to larger plaques under methylcellulose and thus were ideal for fluorescence imaging. Virus stocks of the KOS strain of HSV-1 and the recombinant viruses were prepared as described previously (Desai et al., 1998). Subconfluent monolayers of Vero cells in tissue culture dishes were co-transfected with KOS infected cell DNA (Person and Desai, 1998) and HindIII linearized plasmid DNA. Three days post-transfection the cells were collected, freeze-thawed once, sonicated, and progeny viruses were plaqued. Single plaque isolates were visualized under a fluorescent microscope to confirm homologous recombination. Fluorescent plaques were picked and then purified using sequential cycles of limiting dilution. The purified viruses were then amplified to produce high titer working stocks.

We analyzed all the different recombinant viruses for growth properties, expression of the fusion polypeptide, capsid as well as virion incorporation of the VP26-FP. First we imaged all plaques that developed during the course of a 3-day incubation under methylcellulose using a fluorescence microscope (Fig. 2A). These images showed how the different fluorescent tags localize in