An infectious recombinant equine arteritis virus expressing green fluorescent protein from its replicase gene

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Thus far, systems developed for heterologous gene expression from the genomes of nidoviruses (arteriviruses and coronaviruses) have relied mainly on the translation of foreign genes from subgenomic mRNAs, whose synthesis is a key feature of the nidovirus life cycle. In general, such expression vectors often suffered from relatively low and unpredictable expression levels, as well as genome instability. In an attempt to circumvent these disadvantages, the possibility to express a foreign gene [encoding enhanced green fluorescent protein (eGFP)] from within the nidovirus replicase gene, which encodes two large polyproteins that are processed proteolytically into the non-structural proteins (nsps) required for viral RNA synthesis, has now been explored. A viable recombinant of the arterivirus Equine arteritis virus, EAV-GFP2, was obtained, which contained the eGFP insert at the site specifying the junction between the two most N-proximal replicase-cleavage products, nsp1 and nsp2. EAV-GFP2 replication could be launched by transfection of cells with either in vitro-generated RNA transcripts or a DNA launch plasmid. EAV-GFP2 displayed growth characteristics similar to those of the wild-type virus and was found to maintain the insert stably for at least eight passages. It is proposed that EAV-GFP2 has potential for arterivirus vector development and as a tool in inhibitor screening. It can also be used for fundamental studies into EAV replication, which was illustrated by the fact that the eGFP signal of EAV-GFP2, which largely originated from an eGFP–nsp2 fusion protein, could be used to monitor the formation of the membrane-bound EAV replication complex in real time.

INTRODUCTION

The possibilities to exploit the genomes of nidoviruses (arteriviruses and coronaviruses) as vectors for heterologous gene expression have been explored with different degrees of success. These enveloped, positive-stranded RNA viruses have two properties that appear very useful for this type of research. First, their relatively large genome (13–32 kb) can tolerate large insertions and, in fact, the acquisition of additional genetic material appears to have played a major role in nidovirus evolution (Gorbalenya et al., 2006). Secondly, nidoviruses produce up to nine subgenomic (sg) mRNAs (Lai & Holmes, 2001; Snijder & Meulenberg, 2001), potentially allowing the expression of multiple heterologous genes (Thiel et al., 2003). The construction of such expression vectors has been facilitated enormously by the development of full-length cDNA clones of several nidovirus genomes (van Dinten et al., 1997; Meulenberg et al., 1998; Almazan et al., 2000; Yount et al., 2000, 2003; Casais et al., 2001; Coley et al., 2005; St Jean et al., 2006).

Thus far, the development of nidovirus-based systems for foreign gene expression has mainly focused on coronaviruses, and heterologous genes were expressed almost exclusively from sg mRNAs. In a number of cases, coronavirus genes encoding so-called accessory proteins, which are expressed from sg mRNAs and are dispensable for replication in cell culture, were replaced by foreign genes to engineer infectious recombinant viruses (Fischer et al., 1997; Curtis et al., 2002; Sarma et al., 2002; Sola et al., 2003). As an alternative strategy, the synthesis of additional sg mRNAs for the expression of heterologous inserts was explored (Zhang et al., 1997, 1998; Stirrups et al., 2000;
Alonso et al., 2002; de Haan et al., 2003). In particular, expression vectors derived from defective coronavirus genomes, which require a helper virus for their propagation, were found to be quite unstable (Enjuanes et al., 2001; Dove et al., 2004). Also, expression vectors based on the full-length coronavirus genome suffered from intrinsic genome instability, displayed a variable (low) level of foreign protein synthesis and exhibited growth defects (Fischer et al., 1997; Hsue & Masters, 1999; Sola et al., 2003).

As a vector, arteriviruses would appear to have a number of disadvantages compared with coronaviruses. Their smaller genome can tolerate only small insertions, e.g. due to packaging constraints imposed by the icosahedral nucleocapsid. In contrast to coronaviruses, the 3′-proximal part of the arterivirus genome does not contain any genes that are dispensable for the production of progeny virions (Molenkamp et al., 2000c; Wieringa et al., 2004; Wissink et al., 2005). Consequently, gene replacement, as was attempted in the past (van Dinten et al., 1997), is not an option unless complementing cell lines or expression systems are used to supply the protein encoded by the deleted gene (Welch et al., 2004; Zevenhoven-Dobbe et al., 2004). Another difficulty when using arterivirus sg mRNAs for heterologous gene expression is the overlapping organization of the structural-protein genes, which hampers the straightforward insertion of foreign sequences (de Vries et al., 2001).

In view of these complications, insertion of foreign sequences in the large arterivirus replicase gene, which is explored in this study, represents an interesting alternative for heterologous gene expression. As in all nidoviruses, the equine arteritis virus (EAV) replicase gene consists of two large open reading frames, ORF1a and ORF1b, encoding the polyproteins pp1a and pp1ab. Expression of the latter ORF involves a −1 ribosomal frameshift (RFS) just upstream of the ORF1a termination codon (Fig. 1a) (den Boon et al., 1991). Proteolytic processing of the EAV replicase polyproteins is mediated by three internal proteinases residing in non-structural proteins 1 (nsp1), nsp2 and nsp4, to produce the functional replicate subunits nsp1–nsp12 (reviewed by Ziebuhr et al., 2000). The papain-like autoproteinase domains in nsp1 and nsp2 mediate the liberation of these subunits from the polyproteins, whereas the chymotrypsin-like nsp4 proteinase is responsible for the processing of all cleavage sites downstream of the nsp2/3 junction [processing of pp1a is summarized in Fig. 1(b)] (Snijder & Meulenberg, 1998; Ziebuhr et al., 2000).

Three sites in replicase ORF1a, located directly upstream of the regions encoding nsp1, nsp2 and nsp4, were tested for their ability to accept an insertion (Fig. 1b). Only when a cassette comprising the enhanced green fluorescent protein (eGFP) gene (729 nt) and the foot-and-mouth disease virus (FMDV) 2A oligopeptide (69 nt) was inserted at the ORF1a position specifying the nsp1/nsp2 cleavage site was a viable eGFP-expressing recombinant virus (EAV-GFP2) obtained, which displayed growth characteristics similar to those of the wild-type (wt) virus. As anticipated (de Vries et al., 2001), the data presented here indicate that the EAV replicase gene can indeed accommodate...
relatively large foreign inserts such as eGFP. Not unexpectedly, however, the position of the insert in the replicase polyprotein proved to be a critical factor for virus viability. The potential applications of EAV-GFP2 in fundamental and applied studies are discussed.

METHODS

Cells, virus infections, passaging and plaque assays. EAV (Bucyrus) and recombinant EAV-GFP2 were both propagated in baby hamster kidney cells (BHK-21), rabbit kidney cells (RK-13) or Vero-E6 cells, essentially as described previously (van Berlo et al., 1982). Serial undiluted passaging was performed essentially as described before, except that cells were initially infected with plaque-purified virus at an m.o.i. of 10 (Molenkamp et al., 2000b). Virus titration using plaque assays was done as described previously (Molenkamp et al., 2000a).

Construction of EAV vectors. We generated four full-length cDNA clones, based on clone pEAV211 (van den Born et al., 2005a), specifying EAV recombinants that contained the eGFP gene inserted into their ORF1a locus (Fig. 1b). To create pEAV-GFP1, a DNA linker encoding the 23 aa FMDV 2A oligopeptide was fused in frame to the 3’ end of the eGFP gene. This eGFP–2A cassette was placed in frame at the 5’ end of the replicase gene and introduced into plasmid pLS-LR (van den Born et al., 2004) by using an engineered Ncol site at nt 302 of the EAV genome (Genbank accession no. X53459), which included a novel replicase translation-initiation codon. Amino acids Met-1 and Ala-2 of the replicase polyprotein were replaced with Pro to promote 2A activity (Fig. 1c). For pEAV-GFP2, the same eGFP–2A cassette was inserted in frame into ORF1a by using an engineered Ncol site at nt 1018 (van den Born et al., 2005a), located five codons downstream of the site that encodes the nsp1/2 junction. Thus, the first five codons of nsp2 were maintained to ensure processing of the nsp1/eGFP–2A junction by the nsp1 autoprotease (Fig. 1c; Snijder et al., 1992). The first amino acid of nsp2 (Gly) was replaced by Pro to promote 2A activity (Fig. 1c). The codon usage for nsp2 residues 2–5 was altered (GGCUACAAUCCCA to CCCUAAUACCCGCCA) to minimize the chance of RNA recombination with the corresponding sequence directly upstream of the eGFP–2A cassette (see above). pEAV-GFP3 is a derivative of pEAV-GFP2 in which the entire 2A-encoding sequence is replaced by the eGFP–2A cassette (see above). For Western blotting, transfected BHK-21 cells were lysed at 15 h.p.t. (de Vries et al., 1992). Proteins were separated by SDS-PAGE (12.5 % gels) and transferred to a Hybond-P PVDF membrane (Amersham Biosciences), essentially as described previously (Snijder et al., 1994). Proteins were detected by using nsp1-specific monoclonal antibody 12A4 (1 : 2000) (Wagner et al., 2003), rabbit sera recognizing nsp2 (1 : 2000) (Snijder et al., 1994), nsp3 (1 : 500) (Pedersen et al., 1999) or eGFP (1 : 2000) and appropriate peroxidase-conjugated secondary antibodies. Protein bands were visualized by using the ECL plus Western blotting detection system (Amersham Biosciences).

Metabolic labelling of intracellular protein synthesis in RK-13 cells with [35S]methionine/[38S]cysteine containing Pro-mix L-35S (Amersham Biosciences) was carried out between 5 and 8 h post-infection (p.i.) as described before (de Vries et al., 1992; Snijder et al., 1994). The immunoprecipitated proteins were resolved by SDS-PAGE (10 % gels) and bands were visualized by phosphorimager analysis (see above).

In vivo imaging of EAV-GFP2 replicase expression. Vero-E6 cells were grown to 70 % confluence in a glass-bottomed 10 cm2 culture dish (MatTek Corporation) and were infected with EAV-GFP2 at an m.o.i. of 10. After 1 h, the inoculum was removed and 5 ml prewarmed Dulbecco’s modified Eagle’s medium containing 2 % fetal calf serum was added. The dish was placed in the 37 °C observation chamber (containing 5 % CO2) of a Leica TCS SP5 confocal microscope operating under the Leica Application Suite Advanced Fluorescence software. From 3 to 18 h.p.i., a z stack of five images (total thickness, 6.5 μm) was recorded every 5 min with the pinhole set at 3 Airy units. A 488 nm laser was used for eGFP excitation and emission was recorded at 500–650 nm. Subsequently, movie frames were created by merging the five images of the z series captured at each time point and were processed into a movie of six frames s-1 by using QuickTime Pro. This movie is available as supplementary material in JGV Online.

RESULTS

Strategies for insertion of the eGFP-encoding sequence into the EAV replicase gene

To explore the possibilities for EAV replicase gene-based foreign gene expression, we sought to engineer insertions that would not interfere with the complex proteolytic maturation of the replicase polyproteins. To achieve high
expression levels, the ORF1a region was preferred over ORF1b, as the expression level of the latter has been estimated to be approximately five times lower due to the RFS required for ORF1b expression (Fig. 1a; den Boon et al., 1991). Four recombinant viruses were designed that each contained the eGFP insert at a different position in ORF1a (Fig. 1b). Two of these recombinant viruses contained a ‘self-cleaving’ eGFP–2A module (see below), which was placed upstream of the sequences encoding nspl (EAV-GFP1) or nspl (EAV-GFP2). The other two were designed to have eGFP either attached to the N terminus of nspl (EAV-GFP3) or released from the nspl–nspl region by nspl-mediated processing (EAV-GFP4).

**Insertion of eGFP at the nspl/nspl junction of the EAV replicase is lethal**

For recombinant EAV-GFP4, the nspl/nspl junction was selected as insertion site because its proteolytic cleavage by the nspl proteinase is relatively slow (Snijder et al., 1994), suggesting that this processing step might be less critical than others and thus increasing the chance that the eGFP insertion would be tolerated. To achieve liberation of the eGFP insert from the EAV-GFP4 pp1a and pp1ab polyproteins, the insert was flanked on both sides by amino acid sequences corresponding to the P5–P6’ residues of the natural nspl/nspl cleavage site (Fig. 1b, c). BHK-21 cells were electroporated with *in vitro*-transcribed EAV-GFP4 RNA. IFA of EAV-GFP4-transfected cells fixed at 15 h p.t. revealed a very weak signal for nspl and also some eGFP fluorescence (data not shown). However, when RNA from a replication-deficient EAV mutant with lethal amino acid substitutions in the RNA-dependent RNA polymerase active site was transfected (the EAV030SGA mutant; van Dinten et al., 1999), some nspl signal was also visible. Thus, even in the absence of genome replication, translation of transfected input RNA can yield some background signal in IFA. EAV-GFP4-transfected cells showed the same background expression level as did the non-viable EAV030SGA mutant, suggesting that this recombinant virus was not viable. This observation was confirmed by the absence of virus in the cell-culture medium when tested by using plaque assays and the lack of detectable viral RNA and protein synthesis in hybridization and Western blot analysis, respectively (data not shown).

**eGFP–2A insertions upstream of nspl or nspl do not interfere with genome replication**

It was not investigated in any detail why recombinant EAV-GFP4 was unable to replicate, but probably the insertion at the nspl/nspl cleavage site interfered with the correct maturation of the viral replicase. In an attempt to avoid such interference, expression of eGFP from the 5’ end of the replicase gene was evaluated (recombinant EAV-GFP1; Fig. 1b). In this case, eGFP would be the first replicase gene expression product, thereby reducing the chance that its presence would interfere with replicase processing and function. Previous studies had shown that, in the context of this strategy, eGFP should probably be liberated from nspl to prevent interference with nspl function, which is critical for sg RNA synthesis (Tijms et al., 2001, 2002). To engineer a ‘self-cleaving’ eGFP module, which would cleave the eGFP/nspl junction, the eGFP C terminus was extended with the widely used FMDV 2A oligopeptide, which mediates a co-translational autoprocessing event at its own C terminus (Fig. 1c; Ryan & Drew, 1994; Donnelly et al., 2001). The translation of the eGFP–2A–replicase fusion gene was initiated from a novel translation-initiation codon 79 nt downstream of the original ORF1a AUG codon (van den Born et al., 2004).

Cells transfected with recombinant EAV-GFP1 showed a strong eGFP signal. The protein largely colocalized with replicase subunit nspl in the perinuclear region of the infected cell (Fig. 2). This localization has been documented extensively as the site where most EAV replicase subunits assemble into a membrane-bound replication complex (van der Meer et al., 1998; Pedersen et al., 1999; Snijder et al., 2001). However, in plaque assays of the medium harvested from cells transfected with EAV-GFP1, infectious progeny virions could not be detected (data not shown).

As an alternative strategy, the eGFP–2A gene was inserted between the nspl- and nspl-encoding regions (recombinant EAV-GFP2; Fig. 1b). To ensure processing of the nspl/eGFP–2A junction by the nspl autoproteinase (Snijder et al., 1992), the context of the native nspl/2 site was retained by N-terminally extending the eGFP–2A cassette with the N-terminal 5 aa of nspl (Fig. 1b, c). EAV-GFP2 expressed eGFP efficiently (Fig. 2) and, in addition, produced infectious progeny virus with a titre of approximately 1 × 10^6 p.f.u. ml⁻¹, which is very similar to the titres produced upon wt EAV infection [see also Fig. 5(c)]. The 2A-mediated processing step at the eGFP–2A/nspl junction was required for the viability of EAV-GFP2, because replacement of the N-terminal 21 aa of the 23 aa 2A sequence by a six-glycine spacer (recombinant EAV-GFP3; Fig. 1b) resulted in a replication-competent recombinant (data not shown). Thus, only two recombinant viruses containing the 2A element at the C terminus of their eGFP insert were able to replicate their genome in BHK-21 cells and, of these, only EAV-GFP2 was able to produce infectious progeny.

**sg RNA synthesis is reduced in EAV-GFP1**

To assess whether differences in RNA synthesis could account for the observed phenotypic difference between EAV-GFP1 and EAV-GFP2 (non-infectious versus
infectious, respectively), viral RNA synthesis was evaluated by hybridization analysis of intracellular RNA isolated from transfected cells. By using a probe complementary to the 3' end of all viral RNAs, the accumulation of genome RNA was found to be similar, but EAV-GFP1 produced approximately four times less sg RNA than did the wt control, whereas EAV-GFP2 produced a wt-like RNA pattern (Fig. 3, left panel). A probe complementary to the 3' end of the eGFP gene confirmed the presence of this gene in the EAV-GFP1 and EAV-GFP2 genome RNA (Fig. 3, right panel). No virus-specific RNAs were detected in samples from cells transfected with EAV-GFP3 and EAV-GFP4 (data not shown), indicating that these recombinants indeed failed to replicate their genome. The detection limit of this method is such that input RNA is not detected, as was confirmed by using the non-replicating EAV030SGA mutant (data not shown). In conclusion, the inability of EAV-GFP1 to produce infectious progeny may be due to a defect in sg RNA synthesis, although it certainly cannot be excluded that other defects may have contributed to the observed phenotype, e.g. interference of the inserted sequence with genome packaging.

**Partial release of eGFP–2A in EAV-GFP2 due to incomplete 2A-mediated processing**

The presence of the eGFP–2A insertion in the EAV-GFP1 or EAV-GFP2 replicate polyproteins could affect the proteolytic processing of their N-terminal domains. To evaluate this aspect, lysates from transfected BHK-21 cells were analysed by Western blot analysis using antisera specific for eGFP, nsp1, nsp2 and nsp3. For the non-viable EAV-GFP3, EAV-GFP4 and EAV030SGA mutants, specific but only very faint bands were detected, which was interpreted to be derived from the translation of input RNA (data not shown; see above). For the replication-competent recombinants EAV-GFP1 and EAV-GFP2, it was found that the respective eGFP–2A/nsp1 and eGFP–2A/nsp2 junctions were processed only partially by the activity of the 2A sequence (Fig. 4a, second lane of panel 1 and third lane of panel 2, respectively). When the band intensity of the unprocessed fusion protein (eGFP–2A–nsp1 or eGFP–2A–nsp2) was compared with that of the processing products, it was estimated that the 2A-mediated processing efficiency was not >50%. In both recombinants, all other junctions upstream of nsp4 were cleaved efficiently to yield the usual processing products (Fig. 4a). Although free eGFP–2A was detected on the EAV-GFP1 blot when using the anti-eGFP antiserum, this protein could hardly be detected in the EAV-GFP2 lysate (Fig. 4a, asterisk in third lane of panel...
4). However, in subsequent infection experiments with EAV-GFP2, 35S-labelled eGFP–2A could be immunoprecipitated readily with both anti-eGFP and anti-2A antibodies at an earlier time point (8 h p.i.; Fig. 4b). The apparent loss of eGFP–2A later in EAV-GFP2 transfection/infection was not pursued further, but our findings suggested that eGFP–2A was either unstable in EAV-GFP2-infected cells or may in part have been released from dying cells (see also below and supplementary material, available in JGV Online). In conclusion, although eGFP–2A was released only partially due to the relatively low activity of the 2A element, the eGFP–2A insertion did not interfere with the proteolytic processing of other junctions in the EAV-GFP1 and EAV-GFP2 replicase polyproteins.

EAV-GFP2 replication is somewhat delayed, but the virus is stable upon repeated passaging

To assess the genetic stability of the infectious EAV-GFP2 recombinant, it was analysed in more detail during 15 serial undiluted passages in two independent experiments that started with a plaque-purified virus (defined as passage 1). After each passage, expression of the nsp3 replicase subunit, as detected by IFA, was used as a marker for virus replication. The stability of the eGFP insert was assessed by monitoring eGFP expression in the same cell cultures. All nsp3-positive cells were also positive for eGFP until passage 10 (P10) or P8 in experiment 1 or 2, respectively, after which the number of eGFP-positive cells declined to almost zero during the subsequent five to seven passages (Fig. 5a). To determine whether the loss of eGFP expression was due to the emergence of viruses containing mutations in the eGFP gene, intracellular RNA was isolated after infection with P4, P9 and P14 virus and subjected to RT-PCR and direct sequence analysis. The intact eGFP–2A gene was detected in both P5 samples and in P10 of experiment 1. However, in P10 of experiment 2 and P15 of both experiments, the same predominant deletion mutant was detected, containing an in-frame deletion of the entire eGFP gene and the N-terminal two codons of the 2A element (Fig. 5b). It should be noted that high-titre stocks could be produced routinely from virus stocks with a low passage number and that, in these stocks, EAV-GFP2 always retained its eGFP gene, indicating that the deletion observed upon prolonged passaging is a low-frequency event.

The growth characteristics of EAV-GFP2 were evaluated in a time-course experiment and were compared with those of the wt virus. Growth of EAV-GFP2 was found to be somewhat delayed and its peak titre was slightly lower than that reached by the wt virus (Fig. 5c). This property was also reflected in the somewhat reduced plaque size of EAV-GFP2 (Fig. 5d). In summary, these results indicate that – at least during propagation in cell culture – EAV-GFP2 is reasonably stable and that its growth kinetics are similar to those of the wt virus.

Development of a DNA launch vector for EAV-GFP2

EAV-GFP2 was used to develop a DNA launch system for recombinant EAV. Such a system would circumvent the need for transfection of in vitro-transcribed full-length RNA and takes advantage of RNA polymerase II-driven transcription in the nucleus of the host cell to produce the full-length RNA from a transfected plasmid DNA (Dubensky et al., 1996; Diciommo & Bremner, 1998; Varnavski et al., 2000; Lee et al., 2005). After transfection of a full-length EAV cDNA sequence downstream of a
suitable promoter, the EAV genome RNA would be produced and processed in the nucleus, transported to the cytoplasm and translated to initiate the virus replication cycle.

The RSV promoter was placed directly upstream of the EAV-GFP2 cDNA sequence, as transcription from this promoter starts with a guanine (Yamamoto et al., 1980), which is also the first ribonucleotide of the EAV-Bucyrus genome (van Dinten et al., 1997). For transcription termination and polyadenylation, the SV40 polyadenylation signal was placed downstream of the EAV-GFP2 sequence (Connelly & Manley, 1988). BHK-21 cells were electroporated with the pDE-GFP2 DNA launch plasmid and examined for green fluorescence at several time points after transfection. Three independent experiments were performed and produced similar results. The first cells became eGFP-positive around 20 h p.t. At these early time points, low virus titres ($10^2$–$10^3$ p.f.u. ml$^{-1}$) could be measured, suggesting virus production by just a few cells in the dish. Virus spread to neighbouring cells was observed by 40 h p.t. and eventually resulted in the complete infection of the cell monolayer. Transfection efficiencies were monitored by using a GFP-expressing control plasmid and were found to be at least 80%. These results indicated that EAV-GFP2 replication can indeed be launched by nuclear transcription of transfected plasmid DNA, but the small number of positive cells in the first cycle suggests that the efficiency of this process, or of the subsequent RNA processing and transport steps, is very low. Nevertheless, the system may be useful as a simple procedure to launch recombinant EAV, in particular when subsequent amplification of the recombinant virus is the primary objective.

Visualization of EAV-GFP2 replicase expression by in vivo microscopy

All mammalian positive-strand RNA viruses investigated thus far induce membrane-associated replication complexes in the cytoplasm of the infected cell (Salonen et al., 2005). In EAV-infected cells, nsp2 and nsp3 induce the formation of double-membrane vesicles (DMVs) in the perinuclear region. Although many details of this process remain poorly understood, EAV RNA synthesis appears to be associated with these structures (Pedersen et al., 1999; Snijder et al., 2001). EAV-GFP2 may be a suitable tool to monitor and study replication-complex formation in living cells. The eGFP signal largely colocalizes with that of the viral nsps (Fig. 2) and electron-microscopy studies confirmed that EAV-GFP2-infected cells contain the typical DMVs also observed in cells infected with the wt virus (Fig. 2). Immunolabelling experiments confirmed that these DMVs could be labelled with antibodies recognizing both eGFP and nsps (data not shown).

Between 3 and 18 h p.i., EAV-GFP2-infected cells were monitored by confocal fluorescence microscopy at 5 min intervals. The recorded eGFP images were assembled into a movie documenting the initial phase of replicase
expression and the development of the EAV replication complex (see supplementary material, available in JGV Online). The early eGFP signal localized to defined spots that were interpreted to be the likely sites of initial DMV formation. In the course of the infection, the number of spots increased and they moved to the perinuclear area to form larger clusters and, ultimately, a dense ring surrounding the nucleus in many cells. During the in vivo time-lapse experiment, we also observed a gradual increase of cytosolic eGFP signal, probably representing the cleaved eGFP–2A. Necrosis at later time points (from approx. 14 h p.i. onwards) resulted in release of soluble eGFP–2A into the medium, whereas the perinuclear eGFP staining remained with the remnants of the lysed cell. This is in agreement with our Western blot analysis (Fig. 4a), in which only small amounts of eGFP–2A could be detected at 15 h p.t.

**DISCUSSION**

The present study describes the successful expression of the eGFP gene from the arterivirus replicase gene without compromising the infectivity of the recombinant virus. Previously, the gene conferring resistance to neomycin (neoR) was expressed from the replicase locus of a human coronavirus 229E (HCoV-229E) replicon that was non-infectious due to the absence of several structural-protein genes (Hertzig et al., 2004). In contrast to our EAV-GFP2 construct, the replicase gene of this HCoV-229E replicon was bicistronic, with the most 5′-proximal cistron encoding an nsp1–2A–NeoR protein, the translation of which was initiated from the original replicase start codon. The downstream cistron encoded the other 15 nsps (nsp2–nsp16) and was preceded by an encephalomyocarditis virus internal ribosomal entry site.

Even upon serial undiluted passaging, which often promotes the formation of defective viral genomes, EAV-GFP2 deletion mutants did not emerge within the first seven passages, with the 24 h passaging steps each essentially comprising two EAV replication cycles. A feature that may have contributed to EAV-GFP2 stability is the fact that the eGFP-encoding sequence was part of the replicase reading frame, obviously resulting in a selection pressure to maintain the reading frame and ensure the expression and correct processing of replicase components.

The presence of the 2A element was crucial for EAV-GFP2 replication, as its replacement by a six-glycine spacer (construct EAV-GFP3) was lethal. This result suggested that the release of nsp2 from the eGFP–2A–nsp2 fusion protein was essential for the viability of the recombinant virus. However, nsp2 release by EAV-GFP2 was suboptimal, as the activity of the 2A element in this context was estimated to be <50%, lower than that reported for most other systems in which the 2A oligopeptide has been used (de Felipe et al., 2006). Apparently, the pool of fully cleaved nsp2 was sufficient to support EAV-GFP2 replication and the presence of uncleaved eGFP–2A–nsp2 did not interfere. This might be different for the non-infectious EAV-GFP1 recombinant that showed a defect in sg mRNA synthesis. Here, the uncleaved eGFP–2A–nsp1 fusion protein might have interfered with the crucial function of nsp1 in sg mRNA synthesis (Tijms et al., 2001) or, alternatively, the level of free nsp1 was too low to support wt levels of sg mRNA synthesis. The 2A processing activity can be optimized further (de Felipe et al., 2006), which may be necessary for applications that would require a fully cleaved fusion protein.

The straightforward detection of virus infection by eGFP fluorescence makes EAV-GFP2 a convenient tool for both fundamental and applied research. The potential for use in live microscopy of infected cells is demonstrated (see supplementary material, available in JGV Online) and will be explored in more detail in the context of our studies into the structure and function of the nidovirus replication complex. As EAV-GFP2 was shown to be a suitable vector for the intracellular expression of a foreign gene from the nidovirus replicase locus, this approach may also have potential for vaccine development. Furthermore, the production of an infectious recombinant arterivirus that can be grown to high titres and expresses a reporter gene may facilitate the screening of inhibitors of virus replication in cell culture, as we have demonstrated in a previous study (van den Born et al., 2005b).

**ACKNOWLEDGEMENTS**

We thank Erik van den Berg and Karin Wever for technical assistance during plasmid construction. The anti-2A and anti-eGFP polyclonal antibodies were kindly provided by Dr Marius Ryan (University of St Andrews, Scotland, UK) and Dr Frank van Kuppeveld (Radboud University, Nijmegen, The Netherlands), respectively. We acknowledge the assistance of Anneke Brouwer and Dr Roeland Dirks (Department of Molecular Cell Biology, LUMC) with live imaging microscopy and the general support of Dr Miek Mommaas, Dr Henk Koerten and Dr Willy Spaan.

**REFERENCES**


E. van den Born and others


