ZKBS
Zentrale Kommission für die Biologische Sicherheit

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General position statement of the ZKBS on frequently carried out genetic engineering operations based on the criteria of comparability:

Genetic engineering operations with Sindbis virus and Semliki Forest virus expression systems

1. Sindbis virus and Semliki Forest virus expression systems

1.1. General introduction

SINV and SFV

Both *Sindbis virus* (SINV) and *Semliki Forest virus* (SFV) belong to the family *Togaviridae*, genus *Alphavirus*. The viruses are widespread and transmitted by mosquitoes, SINV by the genus *Aedes*, SFV by the genus *Culex*. In humans both viruses can cause fever. Additional symptoms of a SINV infection are rash and arthritis. The original SFV isolate L10 is neurovirulent in mice, while the SFV isolate A7 is non-virulent [1; 2]. In general, these viruses show a low pathogenicity, although one lethal case of a laboratory infection with SFV was reported [3]. *In vitro* the viruses infect a wide spectrum of hosts, including insects, birds and mammals. Most vertebrate cells are infected lytically, while in insect cells the infection is usually persistent [2]. SINV and SFV are assigned to **risk group 2** according to § 5 Paragraph 6 of the Genetic Engineering Safety Regulations (GenTSV).

The viruses comprise an icosahedral nucleocapsid containing the genome as a single-stranded linear RNA with positive polarity, which is surrounded by a lipid coat embedded with viral coat proteins. The viral genomes have a cap structure at the 5' end and are polyadenylated at the 3' end. Without these terminal structures the respective genome sizes are 11703 nt (49S RNA) for SINV and 11442 nt (42S RNA) for SFV. The genomic RNAs contain two open reading frames, one for the non-structural proteins and one for the structural proteins. Untranslated (UT) nucleotide sequences, which are essential for replication and translation, are located at the termini and between the two reading frames. The packaging signal ψ lies within the reading frame of the non-structural proteins (Fig. 1) [2; 4; 5]. An internal promoter (subgenomic promoter, P_{sg} or 26S promoter) for the (-)-strand RNA is located between the two reading frames.

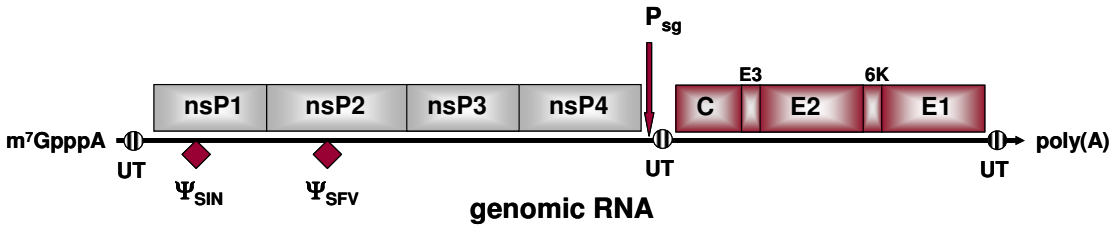

Figure 1: Genome map of SINV and SFV

m^7GpppA: cap structure; ψ: packaging signal; nsP1-nsP4: non-structural proteins; P_{sg}: position of the subgenomic promoter; C: capsid protein; E1, E2, E3, 6K: glycoproteins; Poly(A): polyadenylation; UT: untranslated region.

SINV and SFV replication cycle

Following infection of a cell, the viral genome serves as mRNA. This mRNA is translated into a polyprotein containing the viral non-structural proteins nsP1-4. This precursor protein is processed into the individual non-structural proteins nsP1, nsP2, nsP3 and nsP4 by the protease activity of nsP2. These proteins show the following activities: methyl- and guanylyltransferase in nsP1; protease and RNA helicase in nsP2 and RNA polymerase in nsP4. The function of the phosphoprotein nsP3 is as yet unknown. The non-structural proteins assemble into the replicase and transcribe the (+)-strand genome into the complementary (-)-strand RNA. The (-)-strand serves as the template for producing full-length genomic RNA, as well as for producing the subgenomic 26S RNA from the internal subgenomic promoter. Viral RNA replication takes place in the cytoplasm [6-8].

The subgenomic RNA is also translated into a polyprotein, which is processed by proteolysis into the capsid protein C, the glycoproteins E1, E2 and E3 as well as the 6K protein. The capsid protein is an autoprotease that cleaves itself from the other structural proteins and subsequently recognizes and binds to the packaging signal ψ. This binding leads to packaging of the viral RNA genome into nucleocapsids. The packaging signal is located within the nsP1-coding region in the SINV genome [4], and within the nsP2-coding region in the SFV genome [5]. At the start of infection, E1 is responsible for fusing the viral membrane with the host cell membrane. It forms heterodimers with E2, three of these dimers forming a spike in the viral coat. During assembly of the virion, E2 interacts with the nucleocapsid. The protein E3 is a leader protein, which initially remains attached to E2 in the form of a precursor protein (PE2 or p62). This precursor protein is only cleaved by a cellular protease upon assembly of the new viral particles. In contrast to SFV, no E3 is found in the virus coat of SINV, although both viruses are able to incorporate the uncleaved precursor protein into the virus coat. However, its proteolytic cleavage is a prerequisite for further infectivity [9; 10]. 6K is a leader protein and important for the assembly of viral particles. New viral particles are released from infected cells through budding [2].

1.2. Gene transfer using SINV- and SFV-derived vectors

One can distinguish between three types of SINV- or SFV-derived vector systems.

- Plasmids possessing *cis*-regulatory nucleotide sequences for replication and a promoter for expressing the heterologous gene. They require a helper function to provide non-structural proteins for RNA replication and expression of the heterologous gene as well as structural proteins for particle formation.
- Double promoter plasmids contain an insertion site for a heterologous gene in addition to the reading frames of non-structural and structural proteins. Both the late genes and the heterologous gene are under the control of the subgenomic promoter. These vectors are replication-competent and can package newly produced RNA into viral particles.
- In the third group of vectors, the reading frame of the structural proteins in the original genome is replaced by the heterologous gene. Although the remaining recombinant RNA genome with the reading frame of non-structural proteins can still replicate, no new viral particles can be formed. If the viral structural proteins are provided by a helper function, the replicon can be packaged into replicon particles.

Since the third group of vectors is the most important, only this group of vectors will be discussed in detail in the context of this general position statement.

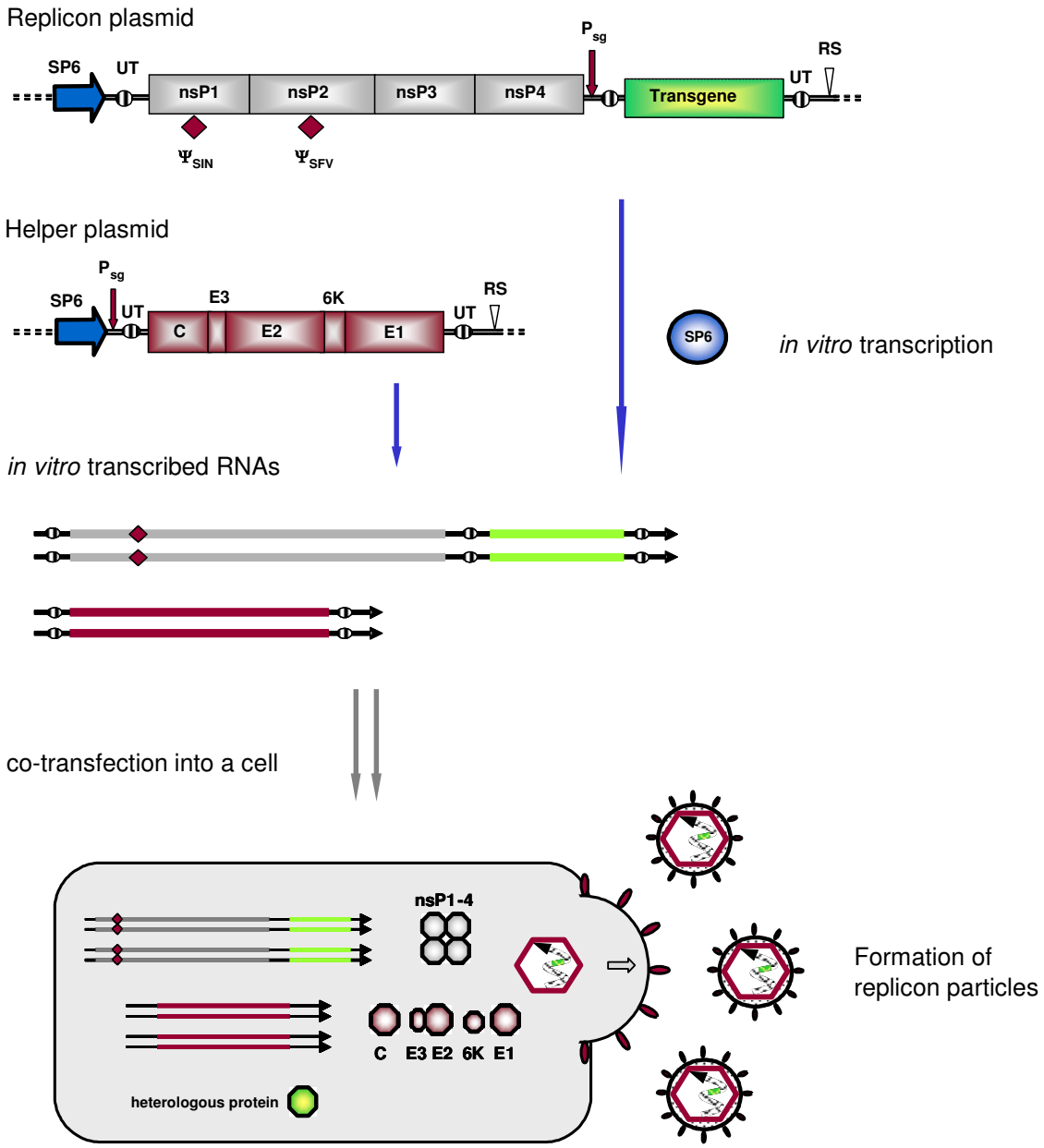

Figure 2: Production of SINV and SFV replicon particles

In the replicon and helper plasmids the viral reading frames are under the control of the SP6 promoter (SP6). The plasmids also contain functions necessary for replication in *E. coli* K12. Plasmid sequences are indicated by dotted lines. The plasmids are linearized at a restriction site (RS) and transcribed *in vitro* by SP6 polymerase (SP6). The *in vitro* transcribed RNAs are co-transfected into a eukaryotic cell where replication of the RNA and expression of viral proteins and the heterologous protein occur. The replicon RNA is packaged into replication-defective viral replicon particles using the structural proteins. Other abbreviations as in Figure 1.

Generating viral replicon particles

To produce viral replicon particles, a cDNA comprising the reading frame of the viral non-structural proteins is placed under the control of a bacteriophage SP6 promoter and inserted in a pUC-derived plasmid (Fig. 2). In addition to the reading frame of the non-structural proteins, this cDNA also carries the viral *cis*-regulatory elements required for replication and translation, as well as the viral subgenomic promoter upstream of the insertion site for the

heterologous gene. To linearize the plasmid, a restriction site is located downstream of the viral cDNA. The replicon RNA (replicon and heterologous gene) is generated *in vitro* using SP6 polymerase, and transfected into a host cell where non-structural and heterologous proteins are expressed. The non-structural proteins replicate the replicon RNA and the subgenomic RNA. To package the replicon RNA into viral replicon particles, structural proteins are translated from a co-transfected helper RNA. The helper RNA is similarly transcribed *in vitro* from a linearized plasmid containing the cDNA of the structural proteins' reading frame under the control of a SP6 promoter. Helper cell lines also exist that constitutively express the structural proteins [11]. The capsid protein recognizes the packaging signal ψ on the replicon RNA and both together form the nucleocapsid. At the cytoplasmic membrane the nucleocapsid is coated in a lipid membrane containing the glycoproteins E1 and E2 protruding as spikes, and released by budding from the cell (Fig. 2). If the *cis*-regulatory sequences are unaltered with respect to the wild-type virus [12; 13] and if vertebrate cells are infected, cytolysis will occur after several days.

Alternatively, the cDNA with the genes for the non-structural proteins and the heterologous gene can be under the control of a polymerase II promoter, as can the cDNA encoding the structural protein genes. In contrast to the system described above, the replicon and helper RNAs are then produced by the host cell itself.

The replication-defective viral replicon particles can be used to infect further host cells in order to express the heterologous gene in these cells. In this case, normally no viral particles are produced, since there are no genes for structural proteins present in the replicon plasmid or the new host cell. These host cells often die after a few days if the *cis*-regulatory sequences are unaltered with respect to the wild-type virus genome and if the host cell is a vertebrate cell.

Prototype plasmids

Prototype plasmids containing the replicon cDNA are pSINrep5 or pSFV1-3. The cDNA of the structural proteins is encoded by prototype helper plasmids pDH-BB (defective helper) or pSFV-Helper1 [14; 15]. These plasmids carry an SP6 promoter for the *in vitro* transcription of the viral functions. Prototype plasmids containing the cDNA of the replicon under polymerase II promoter control are pDLTRSIN-ß-gal or pDCMVSIN-ß-gal. Prototype plasmids that carry the cDNA of the structural proteins under polymerase II promoter control are pDLTR-dinsPSIN or pDCMV-dinsPSIN [16].

1.3. Biological safety of SINV- and SFV-derived vectors

SINV- or SFV-derived vectors are distinguished by their wide host range and rapid expression as well as high expression rate of the transferred gene. Moreover, they have the advantage that they can also infect quiescent cells and replicate their RNA genome in the host cell's cytoplasm without passing through a DNA stage. Normally these vectors have a short expression phase and cytolytic potential.

Particular attention has been paid to the safety of SINV- or SFV-derived vector systems because they have so many possible applications both *in vitro* and *in vivo* [17-19].

Designing the helper plasmid

Generally, replication-defective SINV- and SFV-derived replicon particles are generated and used. The replication defect in these replicons is a relevant safety feature. The first investigations therefore concentrated on the possible recombination between the transfected replicon RNA and the co-transfected helper RNA. Experiments with nucleotide sequences corresponding to replicon and helper RNAs of SINV vectors revealed that replication-competent viruses were released following co-transfection with various deleted or mutated defective

SINV RNAs. The RNA genomes of these viruses arose through recombination between the co-transfected RNAs and were sometimes even longer than the wild-type genome [20].

To avoid the formation of infectious, replication-competent viruses, a triple mutation was introduced at the proteolytic cleavage site of p62 in the SFV-derived helper plasmid (pSFV-Helper1; Fig. 3a). The resulting viral particles are not infectious because they incorporate uncleaved p62 into their viral coat (pSVF-Helper2; Fig. 3b). Only activation with α-chymotrypsin converts the viral replicon particles into an infectious form [21]. In the case of recombination between the RNAs, this should prevent the resulting viruses from being infectious and able to replicate. However, infectious revertants with suppressor mutations (second-site escape mutations) have been observed with both SFV and SINV structural proteins that contained mutations at the proteolytic cleavage site of p62 or PE2, respectively [9].

In general, suppressor mutations arise very infrequently. Similarly, recombination between co-transfected RNAs is a rare event. Therefore, the probability of both events occurring with one RNA molecule is extremely low. Despite this, further development of the SFV helper plasmid led to separating the reading frame of the capsid protein and the coat proteins E3-E2-6K-E1 onto two independent helper RNAs (two-helper RNA system, Fig. 3c). The translation enhancer (enh) from the capsid protein was inserted before the coat proteins E3-E2-6K-E1 for efficient translation. To be able to remove this for correct formation of the spike proteins, the 2A peptide from *Foot and mouth disease virus* was introduced between the proteins E3-E2-6K-E1 and the translation enhancer. Additionally, the autoproteolytic activity of the capsid protein is abolished by a stop mutation, thus further improving safety [22]. Recombination between the RNAs to generate intact infectious RNA is highly unlikely with these viral particles.

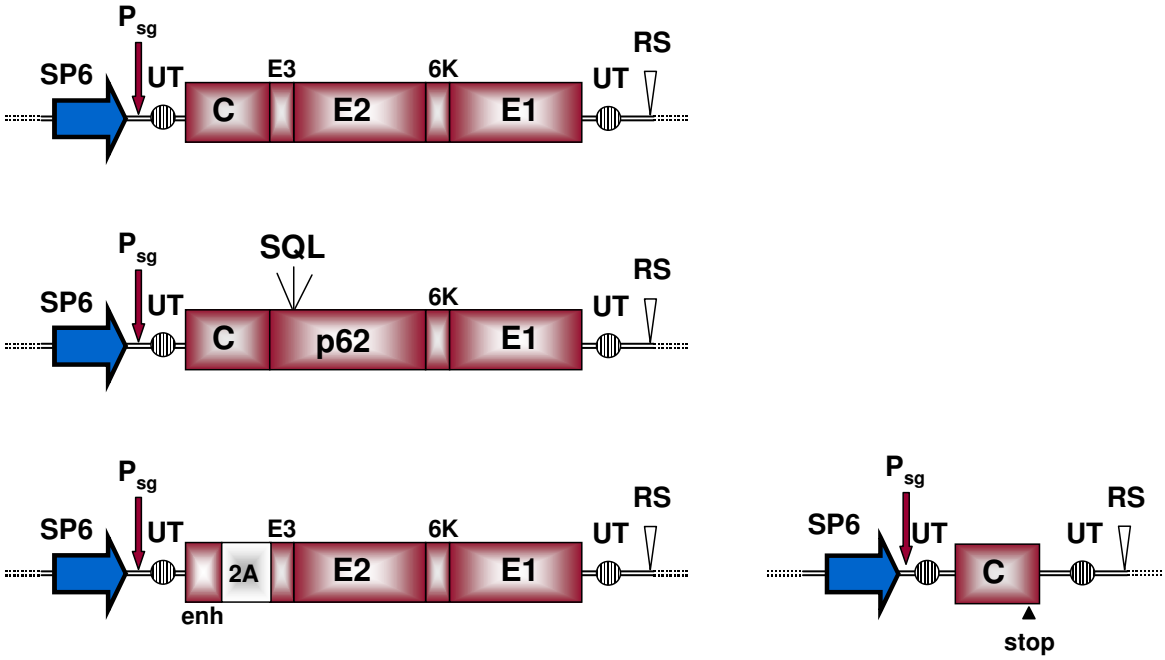

Figure 3: Development of SFV-derived helper plasmids

a The helper plasmid carries the wild-type structural protein genes (pSVF-Helper1).

b Three arginine residues are mutated at the proteolytic cleavage site of p62 (SQL) in the helper plasmid (pSFV-Helper2).

c The genes for the structural proteins C and E3-E2-6K-E1 are located on two independent helper plasmids (two-helper system).
stop: mutation in the capsid gene;
enh: translation enhancer of the capsid protein; 2A: 2A peptide of *Foot and mouth disease virus*.

Other abbreviations as in Figures 1 and 2.

Capsid-independent packaging of the replicon by viral coat proteins

The first step in forming coated RNA viruses and their replicon particle derivatives is the intracellular assembly of genomic or replicon RNA and the capsid protein into a nucleocapsid. This process is mediated by the packaging signal ψ on the RNA. Finally, the interaction between the nucleocapsid and the viral coat proteins leads to the assembly of viral particles.

However, packaging mechanisms with SFV-derived vectors that deviate from this principle have been reported. For example, cells releasing replicating, virus-like particles were observed after infection with a presumed replication-defective SFV vector carrying the glycoprotein gene of *Vesicular stomatitis virus* (VSV-G). These virus-like particles contained the SFV replicon and a membrane coat of VSV-G [23], but were smaller than SFV or VSV particles. This report also mentioned the formation of such replicating particles when an SFV vector was used to express the *Rabies virus* glycoprotein. The plaques formed by these particles in cell culture were smaller than those after VSV-G expression, which means replication proficiencies differ depending on the expressed viral coat protein. The formation of replicating virus-like particles was also described in another report. Here, the SFV vector was used to express the coat protein of *Murine leukemia virus* [24].

Therefore, it cannot be excluded that supposedly replication defective SINV- or SFV-derived vectors can form virus-like particles if the heterologous gene they carry is a gene encoding a viral coat protein.

In analogy to reports of capsid-independent packaging by heterologous coat proteins, the capsid-independent spread of replicon RNA in mammalian and insect cells was shown for SINV- and SFV-derived replicons that encoded the non-structural and coat proteins on a single RNA. Further experiments with the SFV-replicon showed that this spread resulted from the formation and release of infectious virus-like particles. However, these particles proved non-pathogenic in immunocompetent mice after intravenous infection with 10^6 infectious units and despite the detection of infectious material in tissue samples of the heart or lung. Furthermore, there was no evidence that these virus-like particles might have crossed the blood-brain barrier or might have established a productive infection [25].

2. Summary of the relevant criteria for assigning safety and containment levels for genetic engineering operations with Sindbis virus and Semliki Forest virus expression systems

Hazard potential of genetic engineering operations with E. coli K12

Genetic engineering operations with *E. coli* K12 derivatives containing the replicon plasmid including subgenomic nucleic acid fragments or the helper plasmids pose no risk to human health and the environment based on what is currently known in science and technology.

Hazard potential of cells containing a viral replicon

To evaluate the safety of cells acting as recipients for replicon RNA it is important to consider if formation of virus or virus-like particles is possible. Here, it is irrelevant whether the replicon RNA is introduced as *in vitro* transcribed RNA (under SP6 promoter control) or whether it is transcribed from cDNA following introduction into the cell (polymerase II promoter control). As long as no viral coat protein is expressed in the cell, neither replication-competent nor replication-defective viral particles can be formed. Handling such cells is without risk for human health and the environment based on current knowledge in science and technology, as long as the heterologous protein possesses no hazard potential.

It is assumed that the cells referred to above belong to risk group 1. If infection with a micro-organism increases the hazard potential of the cells used, this must be included in the risk assessment. If the cells are infected with viruses or contain viral coat proteins, the hazard potential through an interaction with these viruses or coat proteins must also be taken into account.

Hazard potential of the viral replicon particle

When using a single helper RNA with the complete and unchanged reading frame of the structural proteins, it cannot be excluded that replication-competent, infectious viruses may form due to RNA recombination in cells co-transfected with replicon RNA. The corresponding operations thus pose a small risk for humans and the environment.

When using a single helper RNA with a triple mutation at the proteolytic cleavage site of the E3/E2 precursor protein, handling the replicon particles either before or after their activation with α-chymotrypsin is considered to pose no risk to human health or the environment. Although the replicon particles are infectious after activation, they cannot spread further after the initial infection of a cell.

If the helper function is separated on two helper RNAs it is assumed that the viral replicon particles are replication defective, as long as no heterologous viral coat protein is expressed and there are no homologies in the region of the subgenomic promoter between the replicon-RNA and the helper RNA encoding the alphaviral coat proteins. Infection with replication-defective SINV or SFV replicon particles normally results in cytolysis of the infected cell. The corresponding operations thus pose no risk to humans and the environment.

If the helper function is separated on two helper RNAs and homologies exist in the region of the subgenomic promoter between the replicon-RNA and the helper RNA encoding the alphaviral coat proteins, there is a low probability of an RNA recombination occurring between the RNAs. As a result, RNAs encoding the non-structural as well as the alphaviral coat proteins may arise. Packaging of these molecules would result in the formation of infectious replicon particles that, after the infection of further cells, lead to the capsid-independent formation of replication-competent virus-like particles. Those particles derived from SFV were shown to be non-pathogenic in immunocompetent mice and not sufficient for the establishment of a productive infection [25]. The corresponding operations thus pose no risk to humans and the environment. As yet, the corresponding experimental data for SINV-derived replicons are lacking. However, since a pathogenic potential of SINV-like particles cannot be excluded, the corresponding operations may pose a small risk for humans and the environment.

Hazard potential of the heterologous gene

For viral vectors including the coding sequence of a viral coat protein as the heterologous gene it cannot be excluded that this viral coat protein is incorporated into the coat of the replicon particle or is itself packaging the replicon RNA. In such cases, virus-like replicating particles can be formed upon subsequent infection of other cells. These particles as well as the cells infected by them may possess a low hazard potential. Exceptions are the expression of the coat protein of murine ecotropic retroviruses (not *Lake Casitas virus*) as well as the expression of coat proteins from avian retroviruses of subtypes A and B, since these coat proteins are very labile and have no hazard potential, and their host range is limited to mice and rats, or birds.

3. Criteria for comparability of genetic engineering operations with Sindbis Virus and Semliki Forest Virus expression systems

The following summarizes the general criteria for comparability in genetic engineering operations with SINV and SFV expression systems. The criteria apply to genetic engineering operations with SINV and SFV replicons that carry a heterologous gene with no pathogenic potential. If prion proteins or toxins are expressed, an individual case assessment is required.

Notes

a. The evaluated replicon plasmids are pBR-derivatives carrying cDNA copies of the packaging signal ψ, the *cis*-regulatory sequences for replication and translation of viral RNA, the reading frames of the non-structural proteins and the subgenomic promoter from SINV or SFV. In addition, they possess an insertion site for a heterologous gene and restriction sites for linearizing the plasmid. The plasmids have either a promoter for *in vitro* transcription or a polymerase II promoter to express the viral replicon and the heterologous gene.

b. The evaluated helper plasmids are also pBR-derivatives carrying cDNA copies of *cis*-regulatory sequences for replication and translation of RNA, as well as the reading frames of one or more structural proteins from SINV or SFV. They lack a packaging signal. The plasmids possess either a promoter for *in vitro* transcription or a polymerase II promoter to express the helper RNA.

c. The criteria apply to the introduction of replicon RNA or helper RNA into cells. This can be achieved either by introducing *in vitro* transcribed RNA or by transferring plasmids with the functions listed above as cDNA under the control of a polymerase II promoter.

d. The particular criteria for expression of viral coat proteins do not apply to the coat proteins of murine ecotropic retroviruses (not *Lake Casitas virus*) as well as the expression of coat proteins from avian retroviruses of subtypes A and B.

e. Animals or cells infected with recombinant, replication defective SINV or SFV replicon particles only release recombinant, replication-defective or -competent SINV or SFV particles if:

   - the particles are contaminated with replication-competent infectious viruses,
   - or the recombinant replicon plasmids express a viral coat protein and form virus-like particles.

   The safety measures for handling infected cells or animals are therefore based on the risk group of the recombinant SINV or SFV replicon particles. The infected animals themselves are not GMOs.

   Animals infected with recombinant replication-defective SINV or SFV replicon particles that are assumed not to be contaminated with replication-competent virus or virus-like particles are not GMOs and also not able to pass on GMOs. The animals are carriers of GMOs for a certain period of time.

Introduction of SINV- or SFV-derived replicon plasmids or helper plasmids into E. coli K12

3.1 *E. coli* K12 and its derivatives containing the replicon plasmids with subgenomic viral or cellular nucleic acid fragments mentioned above, or helper plasmids mentioned above are genetically modified organisms of **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

Introduction of replicon RNA into eukaryotic cells

3.2 Eukaryotic cells of risk group 1 containing replicon RNA with a subgenomic viral (except coat protein genes) or cellular nucleic acid fragment are genetically modified organisms of **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

3.3 Eukaryotic cells of risk group 1 containing replicon RNA with a gene of a viral coat protein may release replication-competent, infectious virus-like particles. These cells are genetically modified organisms belonging to **risk group 2**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 2**.

Note: If it is shown that no replicating virus-like particles are formed then the criteria in section **3.2** apply.

3.4 Eukaryotic cells of risk group 2 containing replicon RNA with a subgenomic viral or cellular nucleic acid fragment are genetically modified organisms belonging to **risk group 2**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 2**.

Production of SINV- or SFV-derived replicon particles

3.5 Eukaryotic cells of risk group 1 containing replicon RNA with a subgenomic viral (except coat protein genes) or cellular nucleic acid fragment and additionally a single helper plasmid with the complete reading frame of unmodified SINV or SFV structural proteins release replication-defective, infectious replicon particles. Furthermore, they may release replication-competent, infectious viral particles arising from RNA recombination. These cells are genetically modified organisms belonging to **risk group 2**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 2**.

3.6 Eukaryotic cells of risk group 1 containing replicon RNA with a subgenomic viral (except coat protein genes) or cellular nucleic acid fragment and additionally a single helper plasmid with the complete reading frame of SINV or SFV structural proteins with a triple mutation of the proteolytic cleavage site of the E3-E2 precursor protein release replication-defective, non-infectious replicon particles. Furthermore, they may release non-infectious viral particles arising from RNA recombination. These cells are genetically modified organisms belonging to **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

3.7 Eukaryotic cells of risk group 1 containing replicon RNA with a subgenomic viral (except coat protein genes) or cellular nucleic acid fragment and additionally two separate helper plasmids with the capsid protein and the other SINV or SFV structural proteins that do not show homology to the replicon plasmid release replication-defective, infectious replicon particles. These cells are genetically modified organisms belonging to **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

3.8 Eukaryotic cells of risk group 1 containing SFV-derived replicon RNA with a subgenomic viral (except coat protein genes) or cellular nucleic acid fragment and additionally two separate helper plasmids with the capsid protein and the other SFV structural proteins, with the helper plasmid encoding the coat proteins having homology to the replicon plasmid in the region of the subgenomic promoter, release replication-defective, infectious replicon particles. Furthermore, they may release replication-competent, infectious virus-like particles arising from RNA recombination. These par-

ticles are, however, non-pathogenic. These cells are genetically modified organisms belonging to **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

3.9 Eukaryotic cells of risk group 1 containing SINV-derived replicon RNA with a subgenomic viral (except coat protein genes) or cellular nucleic acid fragment and additionally two separate helper plasmids with the capsid protein and the other SINV structural proteins, with the helper plasmid encoding the coat proteins having homology to the replicon plasmid in the region of the subgenomic promoter, release replication-defective, infectious replicon particles. Furthermore, they may release replication-competent, infectious virus-like particles arising from RNA recombination. These cells are genetically modified organisms belonging to **risk group 2**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 2**.

3.10 Eukaryotic cells of risk group 1 containing replicon RNA with a viral coat protein and additionally one or two helper plasmids with the reading frame of SINV or SFV structural proteins release replication-defective, infectious replicon particles. Furthermore, they may release replication-competent, infectious virus-like particles and replication-competent, infectious viral particles arising from RNA recombination. These cells are genetically modified organisms belonging to **risk group 2**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 2**.

Handling of SINV or SFV replicon particles

3.11 Viral replicon particles generated as described in sections **3.5**, **3.9** and **3.10** are infectious but replication-defective. However, they may be contaminated with replication-competent, infectious viral or virus-like particles. The genetically modified organisms generated in this way belong to **risk group 2**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 2**.

Note: If it is shown that no replication-competent viral or virus-like particles are present then the criteria in sections **3.12** and **3.13** apply.

3.12 Viral replicon particles generated as described in section **3.6** are not infectious before treatment with α-chymotrypsin, and after treatment with α-chymotrypsin are not able to proliferate. The genetically modified organisms generated in this way belong to **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

3.13 Viral replicon particles generated as described in section **3.7** are infectious but replication-defective. The genetically modified organisms generated in this way belong to **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

3.14 Viral replicon particles generated as described in section **3.8** are infectious but replication-defective. However, they may be contaminated with replication-competent, infectious virus-like particles, which are non-pathogenic. The genetically modified organisms generated in this way belong to **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

Infection of eukaryotic cells with viral replicon particles

3.15 Eukaryotic cells of risk group 1 infected by viral particles described in section **3.11** may release replication-competent viral or virus-like particles of risk group 2. The genetically modified organisms generated in this way belong to **risk group 2**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 2**.

3.16 Eukaryotic cells of risk group 1 infected by activated viral particles described in section **3.12** may release viral particles of risk group 1. The genetically modified organisms generated in this way belong to **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

3.17 Eukaryotic cells of risk group 1 infected by viral particles described in section **3.13** do not release viral particles. The genetically modified organisms generated in this way belong to **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

3.18 Eukaryotic cells of risk group 1 infected by viral particles described in section **3.14** may release replication-competent, non-pathogenic virus-like particles of risk group 1. The genetically modified organisms generated in this way belong to **risk group 1**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 1**.

3.19 Eukaryotic cells of risk group 2 infected by viral particles described in sections **3.11** to **3.14** are genetically modified organisms belonging to **risk group 2**. Genetic engineering operations with genetically modified organisms fulfilling these criteria are comparable to each other and are assigned to **containment level 2**.

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