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Examples of expression systems based on animal RNA viruses: alphaviruses and influenza virus

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Successful recovery of RNA viruses and functional RNA replicons from cDNA has greatly facilitated molecular genetic analyses of viral proteins and *cis*-regulatory elements. This technology allows the use of RNA virus replication machinery to express heterologous sequences. Both positive-strand and negative-strand animal RNA viruses have been engineered to produce chimeric viruses expressing protective epitopes from other pathogens and for transient expression of heterologous sequences.

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Introduction

This review looks briefly at the use of animal RNA viruses for expression of heterologous RNAs and proteins. 'RNA viruses', as used here, refers to viruses which have an RNA-only replication strategy, hence excluding the retroviruses. Because of the limited genetic stability of self-replicating RNAs, as a result of both error-prone replication and RNA recombination, it is likely that these RNA-based systems will be most useful for transient expression of RNAs and proteins. This review focuses on systems utilizing alphaviruses and influenza virus as examples of expression systems based upon virus groups with positive-sense and negative-sense replication strategies. A broader review of the subject may be found in [1].

Overview of methodology and terminology

The use of RNA virus replication machinery to express heterologous sequences depends on the ability to engineer recombinant RNA molecules. Although recombinant RNA techniques have been used in studies of the RNA bacteriophage Qβ, the methodology for amplification, *in vitro* cleavage, and ligation of RNA molecules to produce replication-competent viral RNAs remains cumbersome when compared to recombinant DNA technology. Therefore, most studies have relied upon cDNA intermediates that can be used to produce biologically active RNA molecules after either *in vivo* or *in vitro* transcription. As first shown for bromegrass mosaic virus [2], *in vitro* transcription of cDNA templates by *Escherichia coli*, SP6, T7, or T3 DNA-dependent RNA polymerases can generate RNAs with precise 5′ and 3′ termini and specific infectivities that approach those of authentic virion RNAs. RNAs produced *in vitro* can be introduced into cells by microinjection, electroporation, or encapsulation in liposomes. A variety of compounds, such as diethylaminoethyl (DEAE) dextran or cationic lipid micelles, can enhance transfection efficiency. The transfection efficiencies of the different methods vary greatly among cell types, thus limiting the utility of RNA-based expression systems. In some cases, this has been circumvented by devising systems that allow amplification and packaging of recombinant RNAs into infectious virus particles or by expressing replication-competent RNA virus transcripts *in vivo* using recombinant DNA viruses, such as vaccinia (JA Lemm and CM Rice, unpublished data). Alternatively, bacteriophage transcription systems have also been used *in vivo* to produce replication-competent RNA transcripts [3,4•] which allow the use of DNA transfection methodologies.

RNA viruses can be viewed simplistically as consisting of *cis*-acting RNA elements that are necessary for replication, transcription, and packaging, together with genes encoding the viral proteins necessary for these processes. In this review several kinds of recombinant RNA molecules are described. 'Self-replicating RNAs' or 'replicons' will be used to describe RNAs that encode the viral proteins necessary for replication and that also contain the *cis* signals required for replication. RNAs containing the *cis* signals for replication (and in some cases for transcription and packaging) but requiring viral replicase functions to be supplied in *trans* will be referred to as 'defective RNAs'.

Abbreviations

CAT—chloramphericol acetyl transferase; **CTL**—cytotoxic T lymphocyte; **DEAE**—diethylaminoethyl; **DI**—defective interfering; **RNP**—ribonucleoprotein particle; **SFV**—Semliki Forest virus.

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General features of positive-strand RNA viruses

A common property shared by all positive-strand viruses, which has been the key to their development as gene expression vectors, is the ability of naked virion RNA to initiate a productive infection after transfection of an appropriate host cell. The viral genome functions directly as an mRNA for production of all viral proteins necessary for synthesis of a genome-length complementary negative-strand RNA. Once synthesized, this negative-strand template is copied to produce additional genome-length positive strands and, for some viruses, and one or more subgenomic mRNAs. RNA replication and transcription mechanisms are diverse for different positive-strand virus families. For the purpose of discussing the development of these viruses as vectors, two groups can be distinguished based on whether or not subgenomic mRNAs are synthesized. In the group employing the simplest strategy, which includes the picornavirus and flavivirus families, only genome-length positive strands are synthesized. Picornaviruses have been widely used for constructing chimeric viruses expressing short heterologous epitopes [5] and have found more limited use in the production of larger proteins [1,6]. In the other group, which includes the togavirus and coronavirus families, negative strands also function as templates for high level transcription of one or more 3′ co-terminal subgenomic mRNAs. As reviewed below, it is this expression strategy that has facilitated the use of alphaviruses for heterologous gene expression.

Apart from the infectivity of their virion RNAs, other common features of positive-strand RNA viruses make them attractive candidates for the development of RNA-based expression systems. For example, possible unpredictable and deleterious effects of nuclear splicing on expression of heterologous RNAs are eliminated as RNA amplification and transcription occurs in the cytoplasm. In addition, with the exception of the coronaviruses, the genomes of these viruses are small, varying in size between 7.5–14 kb, which facilitates construction and manipulation of cDNA clones, but may also impose size limitations on engineered RNAs when viral packaging systems are utilized.

The alphavirus replication cycle

Sindbis virus and Semliki Forest virus (SFV) are the two best studied alphaviruses [7] and both have been used to express heterologous genes. The alphavirus genome consists of a single-strand RNA molecule, about 12 kb in length, which is capped at the 5′ terminus and polyadenylated at the 3′ terminus. The 5′ two-thirds of the infecting genome RNA is translated to produce polyproteins that are processed by cotranslational and/or post-translational cleavage into four non-structural proteins [8] that are required for RNA replication and transcription. Figure 1 shows the alphavirus RNA replication and transcription strategy. A full-length negative strand is synthesized, which then serves as a template for synthesis of new genomic RNA molecules. The structural proteins are encoded in the 3′ one-third of the genome and are translated from a 3′ co-terminal subgenomic mRNA of about 4.1 kb, which is capped and polyadenylated. This subgenomic mRNA is transcribed from the negative strand at an internal site and is not itself a template for negative-strand synthesis, nor is it packaged into mature virions. Virus budding usually occurs at the cell surface and mature virions are released into the culture fluid.

The RNA signals involved in replication, transcription, and packaging relevant to understanding the various alphavirus expression systems described below. Comparison of several alphavirus nucleotide sequences [9] and more recent mutagenesis studies using Sindbis virus, have defined at least five important *cis*-acting RNA elements in the alphavirus genome. Elements believed to play a role in the synthesis of negative and positive strands, include the 5′ terminal 44 nucleotides [10], a conserved RNA sequence/structure of 51 nucleotides located in the nsP1 coding region [11], and 19 nucleotides immediately adjacent to the 3′ terminal poly (A) tract [12,13]. The signal for specific encapsidation of Sindbis RNAs, as shown by *in vitro* and *in vivo* studies [14], resides in the nsP1 coding region (between nucleotides 746–1226). Finally, a conserved sequence of 24 bases, which overlaps with the end of the non-structural coding region and the subgenomic mRNA start site (sometimes referred to as the 'junction region'), has been shown to function as the core promoter for subgenomic mRNA synthesis in the negative strand [15,16]. Although the core promoter is sufficient for subgenomic mRNA synthesis [16], transcription can be enhanced [17••,18] or depressed [19] by including additional flanking sequences.

Alphavirus-based transient expression strategies

As a transient expression system for heterologous RNAs and proteins, alphaviruses offer several advantages [20]. First, alphaviruses infect and replicate in a broad range of host cells including those of insect, avian, and mammalian origin [21]. Second, high levels of cytoplasmic RNAs and proteins can be expressed using the alphavirus replication machinery. In certain vertebrate cell types, the Sindbis virus replicase/transcriptase can produce an estimated 5×10^5 molecules per cell of the subgenomic mRNA, about 10^7–10^8 molecules per cell of the virion structural proteins, and released virus titers in excess of 10^3 infectious particles per cell (Fig. 1). Third, full-length cDNA clones for *in vitro* transcription of infectious RNA transcripts have been developed for several alphaviruses including Ross River virus [22], SFV [23•], Sindbis virus [19], and Venezuelan equine encephalitis virus [24•]. These clones allow rapid construction of infectious recombinant RNA molecules. Finally, in the case of Sindbis virus, well characterized temperature-sensitive mutations in the viral replication

machinery [25,26] and mutations in the subgenomic promoter (R Raju and H Huang, personal communication) [15,27] can be used to modulate viral mRNA synthesis.

Initial alphavirus expression vectors utilized a small Sindbis virus defective interfering (DI) RNA that contained the chloramphenicol acetyl transferase (CAT) reporter gene, the 5′ and 3′ sequences required for replication, and the encapsidation signal [28]. This engineered RNA could be replicated and packaged in the presence of helper Sindbis virus. CAT production by this system was relatively inefficient and later experiments showed that the level of CAT expression could be significantly enhanced by placing the CAT gene under the control of the promoter for subgenomic mRNA synthesis (Fig. 2a). Constructs of this type can produce substantial amounts of heterologous protein translated from a subgenomic mRNA, but rely on the helper virus encoded products for replication, transcription, and encapsidation.

As an alternative system, a prototype replication-competent, but packaging-defective alphavirus RNA replicon was developed by replacing the Sindbis structural genes with the CAT gene [21] (Fig. 2b, top). Similar approaches have also been used for expression of heterologous genes in plants using tobacco mosaic virus [29] and bromegrass mosaic virus [30]. In cells transfected with this Sindbis recombinant RNA, CAT is expressed rapidly, and up to 10^8 CAT polypeptides are produced per transfected cell after 16–20 hours. CAT expression can be regulated by inclusion of a temperature-sensitive mutation which blocks RNA synthesis. An identical strategy has been used recently to develop vectors based on the SFV replication machinery [31••]. As these RNA replicons do not express structural proteins, the level of heterologous product synthesized is directly related to the transfection efficiency of the recombinant RNA. Conditions for efficient RNA transfection using either cationic liposomes [15,32] or electroporation [23•] have been determined for only a few cell types, limiting the utility of these

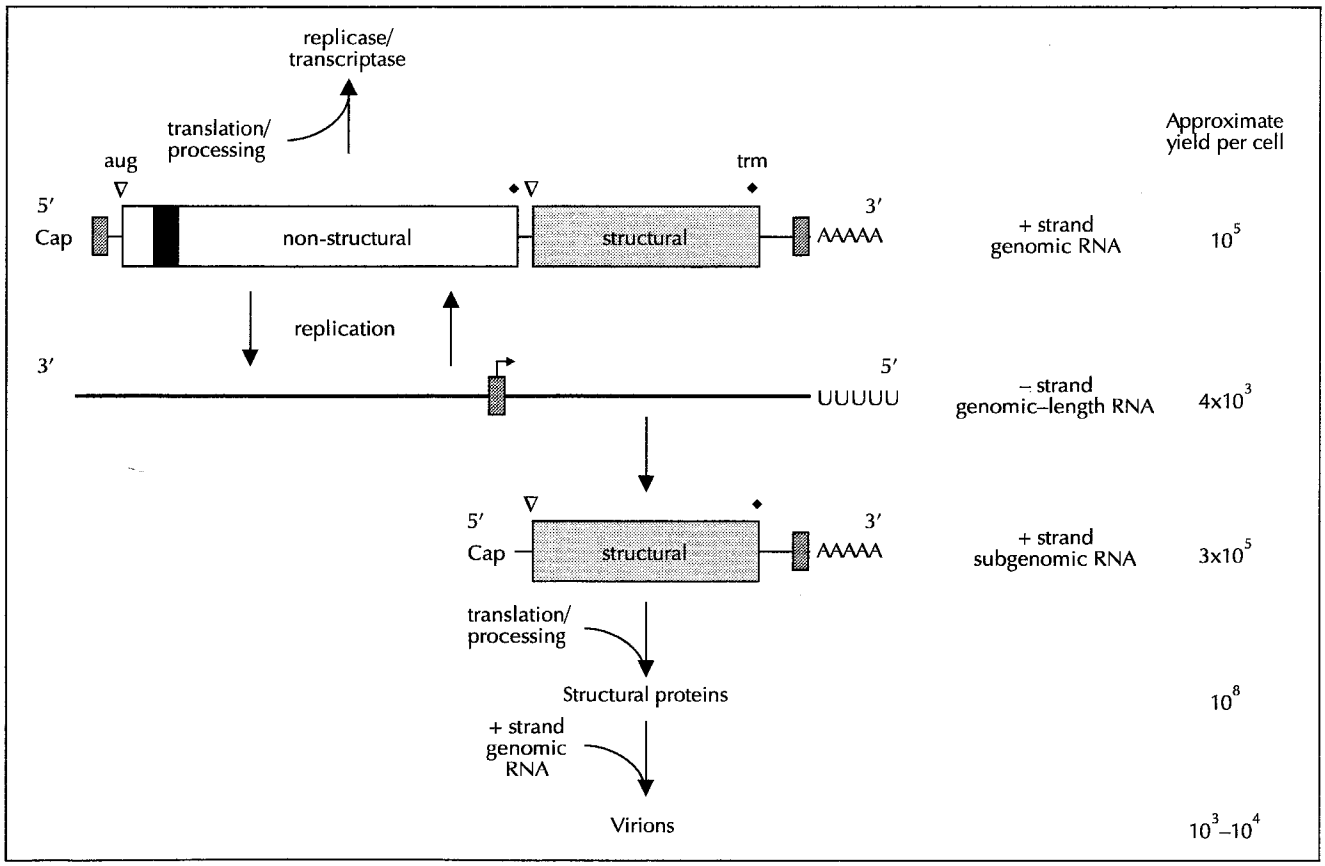

Fig. 1. Alphavirus genome structure and replication strategy. Translated regions of alphavirus genomic and subgenomic RNAs are shown as boxes with the regions coding for non-structural proteins and structural proteins indicated as open and lightly-shaded boxes, respectively. *Cis*-acting sequences important for replication and transcription are shown (small, shaded boxes), and the sequence in the non-structural region important for encapsidation is shown as a black box. The start site for subgenomic mRNA transcription on the negative-strand genome-length RNA template is indicated by an arrow. Translation initiation (aug) and termination signals (trm) are indicated by open triangles and solid diamonds, respectively. The structural and non-structural proteins are processed separately. Non-structural proteins are translated directly from the genomic RNA while structural proteins are translated from a subgenomic positive strand. The full-length genome is replicated and packaged into the structural proteins, forming a mature virion. Approximate levels of virus-specific RNAs and proteins at the peak of virus production in an infected vertebrate cell (permissive for high level virus replication) are indicated on the right. See the text for further details. Adapted from from [1].

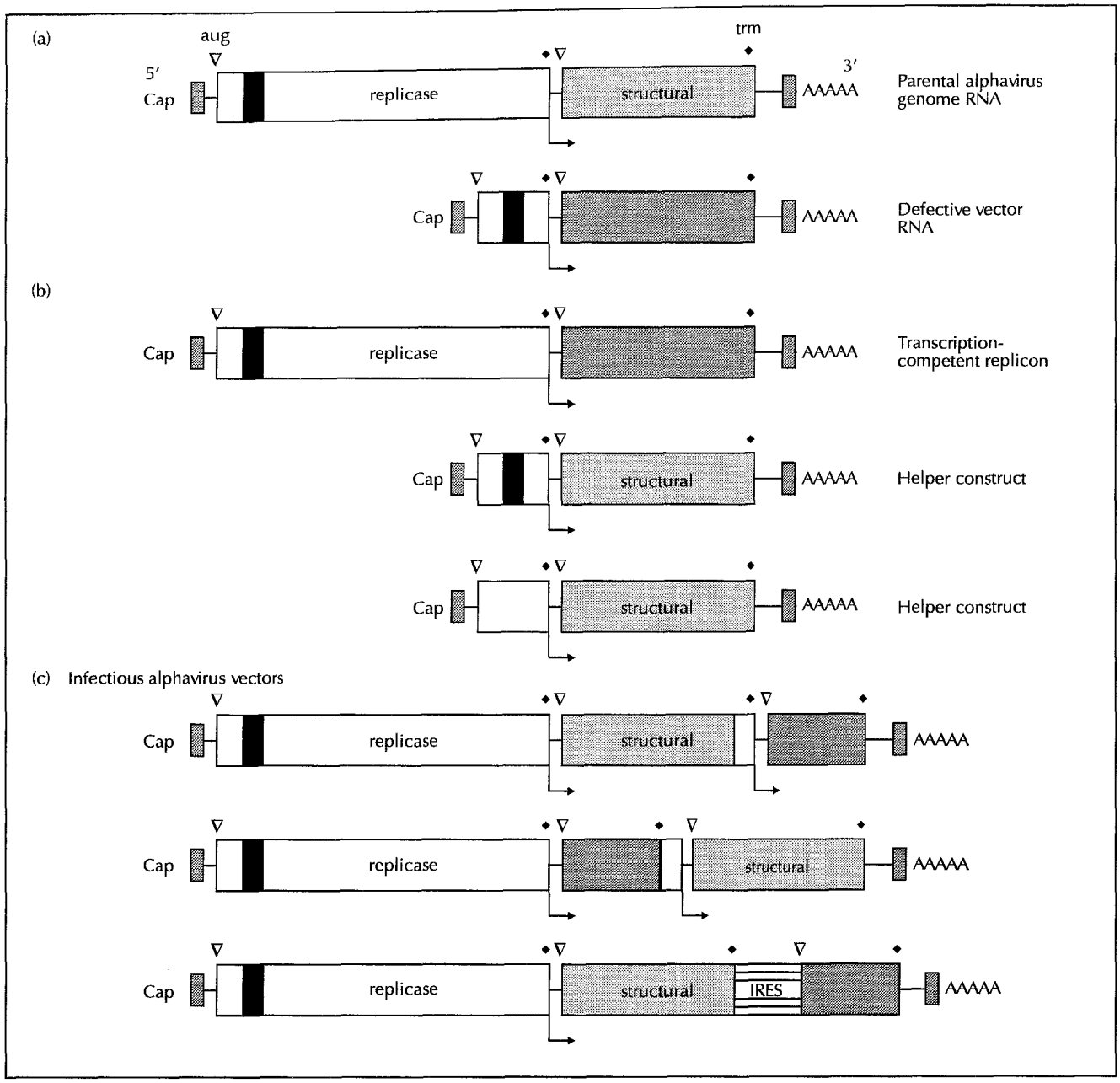

Fig. 2. Alphavirus-derived expression constructs. (a) Structure of the parental alphavirus genome RNA and a defective vector RNA which contains the packaging sequence and expresses a heterologous sequence/foreign gene (darkly shaded box) under the control of a subgenomic mRNA promoter. (b) The top diagram shows a replication and transcription-competent alphavirus RNA replicon expressing a heterologous product via a subgenomic mRNA. The two RNAs shown below are packaging helpers which supply the alphavirus structural proteins after cotransfection with a replicon producing a functional replicase/transcriptase. The first helper construct contains the *cis*-acting signals necessary for replication and transcription, as well as a packaging signal. This results in the production of infectious virus stocks with a bipartite genome structure. The second helper does not contain the packaging signal, allowing the production of helper-free virus stocks of the packaged expression replicon. (c) Infectious alphavirus vectors that contain both the replication machinery and the structural proteins. Heterologous gene products are expressed either by synthesis of a second subgenomic mRNA or by internal translation initiation mediated by internal ribosome entry sites (IRES). For other symbols see Figure 1. See the text for further details. From [1].

vectors to high level production or experiments where expression is required in every cell.

This limitation can be solved by the development of helper systems for packaging the recombinant RNAs into infectious virion particles, with the ideal solution being the production of high titers of helper-free stocks (at least for cell-culture expression studies). Several approaches have been tried. As described for the DI-based vectors, the parental virus can be used as a helper [21]. Alternatively, a construct with the Sindbis structural genes under the control of the subgenomic promoter has been used successfully to supply packaging functions (Fig. 2b) [33••]. In both of these approaches, as the helper RNAs still contain the packaging signal they are also packaged and released as

infectious virions. In the latter case, released virions contain both the vector RNA replicon and the helper RNA and can be propagated serially, resulting in an alphavirus with a bipartite genome structure [33••]. Recently, an efficient helper system has been developed for packaging SFV replicons [31••] that, in contrast to the ones just described, does not contain the region important for alphavirus packaging (Fig. 2b). Packaging of the SFV replicon is achieved by efficient cotransfection of baby hamster kidney cells with both RNAs by electroporation [31••]. Replicase and transcriptase functions supplied by the vector RNA lead not only to its own amplification but also act in *trans* to allow replication and transcription of the helper RNA. This results in synthesis of SFV structural proteins that package the replicon but not the helper, with > 10^8 infectious particles per ml (5×10^9 infectious particles per electroporation) being produced after only 24 hours. Such helper-free stocks can be used, without further phenotypic selection, to infect cells for expression studies or high level protein production. The human transferrin receptor, mouse dihydrofolate reductase, chicken lysozyme, and *E. coli* β-galactosidase have been synthesized successfully using this system with expression levels ranging up to 25% of the total cell protein for β-galactosidase [31••]. It is likely that the packaging capacity of this vector for heterologous RNA will be at least 5 kb.

Although these 'one way' or 'suicide' SFV vectors should in theory be safe for laboratory work, recombination during RNA replication will probably generate infectious SFV at low frequency, as has been observed after cotransfection of similar Sindbis constructs [34]. As SFV can cause serious human disease, it may still be desirable to develop similar systems using less pathogenic or attenuated alphaviruses [24•], or build additional safeguards into the SFV system.

Another strategy (Fig. 2c) for expression of heterologous RNA sequences using alphavirus replication and packaging machinery is the use of recombinant RNAs containing two promoters for subgenomic mRNA synthesis [17••,18]. Heterologous sequences, expressed via a second subgenomic mRNA, can be located either 3′ or 5′ to the Sindbis structural genes. These vectors are both replication and packaging competent and allow the rapid recovery of high titers of infectious recombinant virus stocks, usually in the range of 10^8–10^9 infectious particles per ml. This strategy has been used successfully in a number of expression studies. These include expression of the human tissue plasminogen activator (C Xiong and H Huang, personal communication), drosophila *ftz* protein (E Schroeter and H Huang, personal communication), rubella virus structural genes (T Frey, personal communication), hepatitis delta virus antigen (J Polo, personal communication), and a number of heterologous viral genes (A Grakoui and CM Rice, unpublished data). In addition, the system has been used to express functional murine class I major histocompatability molecules (C Hahn, Y Hahn, V Braciale, T Braciale, CM Rice, unpublished data) and for mapping domains of glucose transporter isoforms important for subcellular localization [35•]. Other studies have shown that recombinants expressing a truncated form of the influenza HA protein or minigenes encoding immunodominant cytotoxic T lymphocyte (CTL) epitopes can be used to sensitize murine target cells for lysis by appropriate class I MHC-restricted CTLs [17••]. Hence, like vaccinia (see Moss, this issue, pp 518–522), this system is useful for rapid mapping and fine-structure analysis of T-cell epitopes expressed via the endogenous antigen presentation pathway. In addition, immunization of mice with the Sindbis double subgenomic vectors [17••] or the bipartite vector (S Virgin and S Schlesinger, personal communication) allows epitope-specific CTL or humoral responses to be primed *in vivo*.

Heterologous polypeptides can also be expressed by using internal ribosome entry sites (see Davies and Kaufman, this issue, pp 512–517) to mediate cap-independent translation initiation on dicistronic mRNA transcripts (Fig. 2c). Using the internal ribosome entry site of encephalomyocarditis virus, CAT expression levels are about five-fold lower than when expression is driven via a second subgenomic promoter (B Pragai and CM Rice, unpublished data).

Some considerations for use of the current alphavirus expression systems are worth noting. For many applications, it will be important for the biology of alphavirus replication to be compatible with the functional expression of the heterologous product in a given host cell. In this regard, it should be noted that alphavirus infection of different cells types can have varied effects on host DNA replication, RNA transcription and mRNA translation [36]. Moreover, recovery of infectious particle stocks may depend on the length or structure of the particular heterologous RNA sequence or an encoded protein product. Interference with alphavirus replication, at any stage in the replication cycle, could preclude the recovery of the desired recombinant. In our experience, packaging constraints on the length of the genomic RNA appear to affect rescue and stability of double subgenomic Sindbis recombinants [1]. Preliminary experiments suggest that double subgenomic vectors with heterologous inserts positioned upstream from the structural protein open reading frame may be more stable, although these constructs produce somewhat lower expression levels (CS Hahn, A Grakoui, C Xiong, H Huang, and CM Rice, unpublished data).

Alphaviruses have also been used as carriers for heterologous polypeptides by incorporation of these sequences into permissive sites in the virion envelope proteins [37•]. In contrast to the approach taken for directed engineering of poliovirus chimeras [5], random insertion mutagenesis has been used to identify insertion sites that allow recovery of chimeric viruses with growth properties similar to parental Sindbis virus. Such sites are found in the virion genes for E1 and E2 glycoproteins and the secreted E3 glycoprotein (S London and CM Rice, unpublished data) [37•]. The use of some chimeras for immunization induces a partially protective immune response against the heterologous

pathogen [37•]. Besides providing a possible approach for developing live-attenuated vaccine viruses, insertion of peptide ligands or larger binding domains into virion surface proteins may ultimately allow delivery of recombinant RNA expression constructs to specific cell types.

General features of negative-strand RNA viruses

Although the negative-strand animal RNA viruses have varied genome structures and host interactions, their RNA replication and transcription strategies are remarkably similar. Key events in the negative-strand virus RNA replication cycle include primary transcription of mRNAs from the entering nucleoprotein core, translation of these mRNAs, replication of the genome RNA(s), secondary transcription and genome amplification, assembly of nucleocapsids, and maturation of enveloped virions by budding. The original distinction between negative-strand and positive-strand RNA viruses [38], relevant to design of expression systems, is that deproteinized genome RNAs of negative-strand viruses cannot initiate a complete infectious cycle after transfection. This observation is due to the negative-sense polarity of their genome RNA(s), that necessitates primary transcription of mRNAs to produce the viral proteins required for subsequent steps in virus RNA replication and virion assembly. Any viral polypeptides required for primary transcription must be present in the entering ribonucleoprotein particle (RNP) and, for most negative-strand viruses, infectious RNPs can be prepared which are devoid of the virion envelope.

Until recently, the development of gene expression systems based on negative-strand viruses has been hampered by a number of technical obstacles. Transfected naked genome or *in vitro* transcribed RNAs, which contain the *cis*-acting signals necessary for transcription, replication and packaging, are generally not rescued by helper-virus infection. Exceptions include a recent report [39•] describing an engineered RNA that can be replicated, transcribed and packaged in the presence of Sendai virus (a paramyxovirus) and the successful recovery of DI RNAs of vesicular stomatitis virus after transfection of DI cDNA clones [4•]. For influenza virus [40,41] and reovirus (a segmented, double-stranded RNA virus) [42], rescue of replication competent RNAs from cDNA has also been demonstrated but requires *in vitro* assembly of an RNP complex prior to transfection. The influenza A virus, Sendai virus and vesicular stomatitis virus systems have been successfully exploited for the expression of heterologous sequences, and it is probable that similar approaches will ultimately be successful for other negative-strand viruses.

Influenza virus-based expression strategies

Details of the influenza virus life cycle relevant to its use for heterologous gene expression are summarized briefly here; a more comprehensive treatment is presented in a recent review by Krug and coworkers [43]. The influenza A virus genome consists of eight RNA segments. Each segment shares common 5′ and 3′ terminal sequences of 15 bases which are complementary and capable of base-pairing to form panhandle structures. The infectious influenza RNP complex consists of these genomic RNAs complexed with nucleocapsid protein and three larger replicase/transcriptase components, PA, PB1, and PB2. A diagram depicting replication and transcription of an influenza segment is shown in Fig. 3a. After entry of the virus, RNA transcription and replication occur in the host cell nucleus. Messenger RNA transcripts are initiated using 10–13-base 5′-capped oligonucleotide primers derived by endonucleolytic cleavage of host RNA polymerase II transcripts. Influenza mRNAs terminate with a 3′ terminal poly (A) tract derived from a short poly (U) template [44]. Transcripts therefore lack the 3′ terminal conserved element found on genome-length RNAs and are transported to the cytoplasm for translation and are not packaged into nucleocapsid structures or virions. Expression of influenza A proteins from segments 7 and 8 involves synthesis of multiple spliced mRNAs suggesting that heterologous RNAs expressed using the influenza virus transcriptase may be targets for the host splicing machinery. Influenza mRNAs are preferentially translated relative to host mRNAs, resulting in a dramatic decrease in host protein synthesis in infected cells [43].

The system developed for recovering influenza A virus gene segments from cDNA is shown in Fig. 3b [40,41,45•]. In the first successful experiments, an engineered RNA segment containing the bacterial CAT-coding region flanked by 22 5′-terminal and 26 3′-terminal bases was synthesized by *in vitro* transcription of a linearized cDNA template using T7 RNA polymerase [40]. These RNA transcripts could be reconstituted into RNPs, which were functional for influenza transcription *in vitro*. When such RNPs were used to transfect influenza-infected MDCK or MDBK cells, CAT activity was detected, indicating that the influenza helper virus was able to replicate and transcribe the engineered RNA segment. Passaging experiments demonstrated that the engineered segment also contained the *cis* elements necessary for packaging. By coupling *in vitro* RNA transcription and RNP formation, the efficiency of this RNP transfection system has recently been improved more than 100-fold [45•].

This technology has had tremendous impact for basic studies on influenza virus replication and may also be useful for developing improved influenza virus vaccine strains. Studies dealing with analysis of influenza *cis*-acting RNA elements [41,44] and engineering of

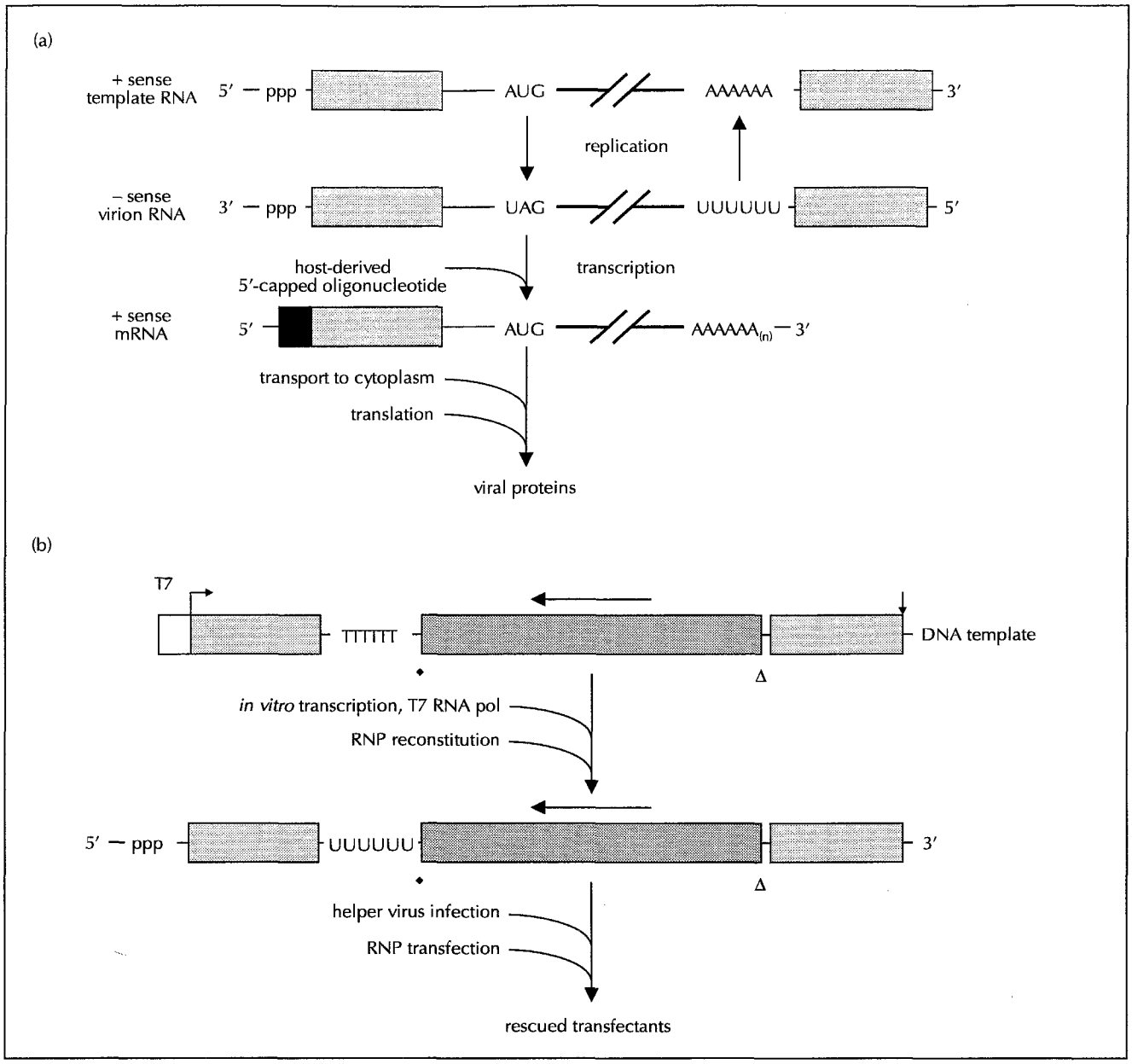

Fig. 3. Influenza A virus. (a) Structure, replication and transcription of an influenza A virus RNA segment. Virion negative-sense RNA, the complementary positive-sense template RNA, and the mRNA transcript are shown. Complementary sequences, present at the 5′ and 3′ termini of genome-length negative and positive-sense RNAs, which can base-pair to form panhandle-like RNA structures, are shown as lightly shaded boxes. The short stretch of poly (U), which apparently serves as the template for polyadenylation of mRNAs by 'polymerase slippage', is shown in the negative-sense virion RNA. Host-derived, 5′-capped oligoribonucleotides, used as primers for transcription of influenza mRNAs, are denoted by a black box. See the text for further discussion of the influenza replication strategy. (b) Methodology for recovery of influenza A virus transfectants. A plasmid construct is made which contains the T7 RNA polymerase promoter upstream from cDNA corresponding to the 5′ and 3′ terminal sequences of the influenza negative-sense virion RNA, denoted as in (a). The cDNA encoding the heterologous sequence or foreign gene (darkly shaded box) is positioned between these *cis* elements in the antisense orientation with respect to the virion RNA transcript. Open triangles and solid diamonds indicate initiation and termination codons, respectively. Run-off RNA transcripts are produced by T7 RNA polymerase after digestion of template DNA at a unique restriction enzyme cleavage site (indicated by an arrow) which immediately follows the 3′ terminus of the virion negative-sense cDNA. Infectious ribonucleoprotein particles (RNPs) are reconstituted from *in vitro* transcribed RNAs and nucleocapsid components are purified either using a two-step procedure [40,41] or a more efficient method where transcription and RNP assembly are coupled [45•]. Reconstituted RNPs are then used to transfect appropriate host cells which have been previously infected with helper influenza virus. This procedure allows replication, expression and packaging of some engineered segments. From [1].

influenza strains (called 'transfectants') [45•] with impaired replication and an attenuated phenotype [46••] or carrying epitopes from different influenza subtypes [47••], have already been reported. CAT remains the only reported example of heterologous gene expression by influenza, and from the published literature it is difficult to determine the actual expression levels on a per-cell basis.

The utility of the system for expression studies directly after transfection is currently limited by the low RNP transfection efficiency (0.2% of the cells productively transfected) [45•]. In theory this could be circumvented by the recovery of pure influenza transfectant stocks which would then allow expression of the heterologous gene at levels similar to those observed for influenza virus gene products. Alternative strategies will need to be employed, however, that allow either substantial increases in rescue efficiency (like *in vivo* transcription/RNP reconstitution) or positive selections for transfectants carrying the heterologous sequence. One possible approach, similar to that used for poliovirus and Sindbis virus, would be to incorporate heterologous segments into non-essential regions of essential influenza gene products, such as the influenza hemagglutinin or neuraminidase. A more generally useful strategy may be to utilize engineered gene segments that are dicistronic, expressing both an essential influenza gene or a dominant selectable marker in addition to the heterologous sequence. As discussed for the alphaviruses, RNA sequences allowing internal translation initiation might allow efficient expression of two polypeptides from a single mRNA transcript. Although the packaging capacity of influenza virions has not been established, a recent study has shown that transfectants with at least nine segments can be recovered, albeit with lower efficiency [48••], suggesting that it may be possible to engineer stable transfectants expressing additional gene products.

Conclusions

As can be seen from the preceding discussion, the development of RNA-based expression systems has just begun. Future expression systems may include not only the ability to regulate RNA synthesis, but also *cis* RNA regulatory elements which will enable conditional control of translational efficiency and modulation of mRNA stability [49]. An exciting prospect is the possibility of engineered packaging systems that allow encapsidation of RNA free of size constraints and delivery of replication competent recombinant RNAs to specific cell types. One could envisage the use of either defined packaging signals and their cognate viral proteins, or designs based on other protein–protein and protein–RNA interactions. In any case, it is likely that both current and future RNA-based expression systems will prove to be valuable for transient expression of proteins and peptides, as well as antisense RNAs, RNA decoys [50], and ribozymes.

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