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Replication of Flock House Virus RNAs from Primary Transcripts Made in Cells by RNA Polymerase II

KYLE L. JOHNSON AND L. ANDREW BALL*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

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To develop vector systems that combine high transcription activity with biologically safe delivery vehicles, we have explored the use of RNA replication to amplify mRNAs, by using flock house virus (FHV) as a model system. The FHV RNA replicase is encoded in the larger of the two segments that comprise the viral positive-sense RNA genome. A cDNA copy of this self-replicating RNA was precisely positioned between a promoter site for cellular RNA polymerase II and a cDNA encoding a self-cleaving ribozyme from hepatitis delta virus. Transfection of this plasmid into cultured BHK cells resulted in prolonged, autonomous FHV RNA replication in the cytoplasm and substantial amplification of the RNA replicon. The replicase also amplified RNA transcribed from a second plasmid of similar design that contained a cDNA copy of the other FHV genome segment. These results constitute a significant step toward the harnessing of nodaviral RNA replication as the basis of a versatile vector system.

To achieve the maximum level of expression for a vectored gene, it is advantageous not only to optimize its *cis*-acting signals but also to use a vector that amplifies both the template and the RNA polymerase that is responsible for its transcription. However, while the ability to replicate and transcribe independently of the cell cycle greatly extends the range of cell types accessible to a vector, these properties also usually render it oncogenic or cytocidal and thereby limit its value for gene therapy. In an attempt to circumvent some of these problems, we and others have begun to explore different ways to amplify mRNA levels directly by using RNA replication. For example, vector systems have been developed from members of the *Alphavirus* genus of the *Togaviridae*, specifically Sindbis virus (30, 33), Semliki Forest virus (25), and Venezuelan equine encephalitis virus (12), and we have examined nodavirus RNA replication from the same perspective.

RNA replication catalyzed by the RNA-dependent RNA polymerase (RNA replicase) of the nodavirus flock house virus (FHV) is unusually vigorous and compatible with a wide range of intracellular environments. Although FHV has a natural host range that is limited to insects (2, 20), its RNAs can replicate in insect (18), vertebrate (5), plant (31), and even yeast (27) cells, suggesting that any host factors involved in RNA replication must be conserved among these widely divergent organisms (1). The catalytic subunit of the RNA replicase (protein A) is encoded by the larger segment of the bipartite positive-sense RNA genome (RNA 1; 3107 nucleotides [nt]), which can therefore replicate autonomously in the cytoplasm, reaching levels as high as those of the rRNAs (1, 5, 18). During RNA 1 replication, a small subgenomic RNA (RNA 3) which contains the 3′ 387 nt of RNA 1 (4, 19) is also synthesized. RNA 3 encodes two small nonstructural proteins of unknown function, B1 and B2 (4, 14, 15), which appear to be dispensable for RNA replication in cell culture (4). Under natural conditions, the replicase copies both RNA 1 and RNA 2 (1,400 nt), which is the genome segment that encodes the viral coat protein precursor. The ability of the FHV replicase to function in *trans* to replicate RNA 2 adds to its attractions as the basis for a vector system, and the *cis*-acting signals of RNA 2 can indeed be used to support the replication and expression of heterologous RNA sequences (27, 35).

Using vaccinia virus (VV)-FHV recombinants or VV-expressed T7 RNA polymerase (17) to drive transcription from T7-FHV plasmids, we found that in order to be competent templates for RNA replication, the primary transcripts must have termini that closely resemble those of the authentic FHV RNAs (1, 3, 4, 6, 7, 24). This was achieved by positioning the promoter precisely at the 5′ end of the FHV cDNA and by site-specific RNA cleavage at the 3′ end of the primary transcript, mediated by the antigenomic ribozyme of hepatitis delta virus (HDV) (26). However, the potential applications of these studies were limited to some extent by their dependence on the cytocidal VV, and to circumvent this problem, we sought ways to generate self-replicating RNAs by using a cellular RNA polymerase. Here we describe the use of RNA polymerase II (pol II) for the intracellular synthesis of primary FHV transcripts that undergo cytoplasmic RNA replication in mammalian cells in the absence of VV infection. This extends the potential applications of the nodavirus system for the development of long-term, high-level expression vectors.

Replication of FHV1 and FHV2 RNA pol II transcripts in transfected cells. As in earlier studies (1), we constructed plasmids that directed transcription of FHV RNA 1 and RNA 2 molecules with authentic 5′ and 3′ termini, but in this case, the FHV RNA 1 or RNA 2 cDNAs were introduced downstream of the simian virus 40 (SV40) early promoter or the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter. The resulting circular plasmids retained the general structure 5′-promoter–FHV cDNA–HDV ribozyme–bacteriophage T7 terminator-3′, linked by vector sequences (pGEM3/4; Promega Biotec). The T7 transcriptional terminator (29) was retained because it appeared to increase ribozyme-mediated cleavage of some transcripts, even though it presumably did not terminate pol II-catalyzed transcription. SV40 nt 5241 to 5236 (8), which encompass the initiation site of the early promoter, were altered from 5′-CCTCGG-3′ to 5′-CTGCAG-3′ to create a *Pst*I site. Similarly, nt 9290 to 9297 of RSV (34), which encompass the initiation site of the LTR promoter, were changed from 5′-AATAAACG-3′ to 5′-CGTCGACG-3′ to form a *Sal*I site.

* Corresponding author. Mailing address: Department of Microbiology, BBRB 373/17, University of Alabama at Birmingham, Birmingham, AL 35294-2170. Phone: (205) 934-0864. Fax: (205) 934-1636. E-mail: andy_ball@micro.microbio.uab.edu.

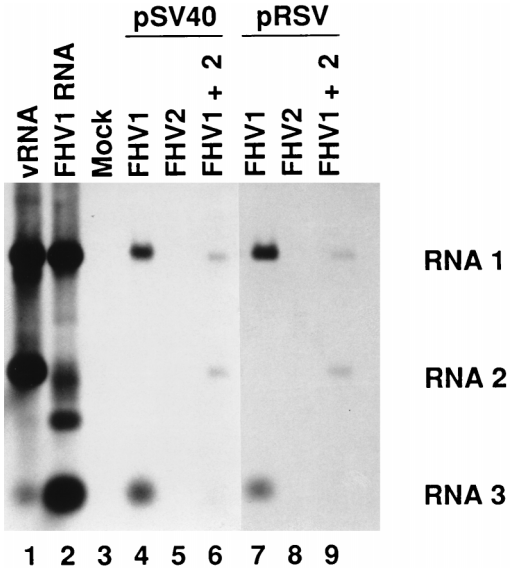

FIG. 1. Products of RNA replication in cells transfected with pol II-FHV transcription plasmids. BHK21 cells were transfected singly with pSV40- or pRSV-FHV1[0,0] or pSV40- or pRSV-FHV2[0,0] plasmids (1.3 μg) or were cotransfected with the two plasmids (1.3 μg each), with Lipofectamine (14 μg) and OptiMEM I (1 ml) (GIBCO/BRL). Using this protocol and a reporter plasmid that contained the *Escherichia coli lacZ* gene driven by the RSV LTR promoter (pRc/RSVβgal; obtained from Henry O. Stone and colleagues, East Carolina University, Greenville, N.C.), we estimated transfection efficiencies at 28°C as approximately 10% (data not shown). The cells were incubated at 28°C for 4 days and then were labeled for 2 h with [^{3}H]uridine in the presence of act D to inhibit DNA-dependent RNA synthesis. Total cellular RNAs were extracted with guanidine thiocyanate (RNAgents; Promega Biotec) (11). Samples corresponding to the RNA from 10^5 cells were resolved by electrophoresis on a denaturing formaldehyde-agarose gel (23) and visualized by fluorography (1). Lanes: 1, RNAs from cells transfected with RNA isolated from purified FHV virions (vRNA); 2, RNAs from cells transfected with purified FHV RNA 1 (FHV1 RNA); 3, RNAs from mock-transfected cells; 4 to 9, RNAs from cells transfected with the plasmids shown.

These changes allowed the FHV cDNAs to be readily positioned such that transcription should initiate at their authentic 5′-terminal nucleotides. The SV40 promoter also differed fortuitously from the wild-type sequence at two positions upstream of the initiation site (T35C and C36T), and the promoter sequence in pSV40-FHV2[0,0] contained a third change, C14T.

The resulting pSV40-FHV[0,0] plasmids contained the promoter-cDNA-ribozyme junction sequences 5′...GGCCGCTG CA**G**T**.....AGGT**/GGG...3′, in which the FHV sequences are in boldface type. The predicted site of transcriptional initiation by pol II is underlined, and the site of ribozyme-mediated cleavage of the RNA transcript is indicated by a slash. Similarly, the pRSV-FHV[0,0] plasmids contained the junction sequences 5′...TACCGTCGAC**GT.....AGGT**/GGG...3′.

Baby hamster kidney (BHK21) cells in culture at 28°C were transfected with the FHV1 plasmids alone, the FHV2 plasmids alone, or the FHV1 and FHV2 plasmids together. Transfected cells were incubated for 4 days at 28°C and labeled for 2 h with [^{3}H]uridine in the presence of actinomycin D (act D) to inhibit DNA-dependent RNA synthesis (1). Total cellular RNAs were isolated and separated on a denaturing formaldehyde-agarose gel, a fluorograph of which is shown in Fig. 1.

When the pol II-FHV1 plasmids were transfected singly, we observed two labeled RNA species (Fig. 1, lanes 4 and 7) that comigrated with authentic RNAs 1 and 3 (Fig. 1, lanes 1 and 2). The resistance of RNA synthesis to act D (a hallmark of RNA-dependent RNA synthesis) and the appearance of RNA 3, which is templated by the negative strand of RNA 1 (14, 16, 19), indicated that the primary FHV1 transcripts were actively replicating. In accordance with this, no labeled RNA was detected in cells transfected with FHV2 plasmids alone (Fig. 1, lanes 5 and 8).

Cotransfection of cells with FHV1 and FHV2 plasmids (Fig. 1, lanes 6 and 9), resulted in the replication of RNA 2 and a decrease in the replication of RNA 1. In addition, RNA 3 synthesis was suppressed, an expected consequence of RNA 2 replication (36). Plasmid cotransfection thus recreated the situation seen during natural infection with FHV. The identities of the RNAs were confirmed by Northern blot analysis; no RNAs were labeled when these experiments were performed at 37°C, at which the FHV replicase is inactive (5) (data not shown).

Mapping of the 5′ termini of RNAs produced in cells cotransfected with FHV1 and FHV2 plasmids. Cells were cotransfected as described for Fig. 1, and the isolated RNAs from 10^5 cells were analyzed by primer extension (6) with an oligonucleotide primer complementary to nt 2819 to 2801 of RNA 1, which anneals 80 to 98 nt downstream of the 5′ end of RNA 3 (4). The high abundance of replicated RNA 1 and RNA 3 allowed us to use primer extension on 18S rRNA as an internal control for quantitation purposes.

A family of three primer extension products was detected in cells transfected with either of the FHV1 plasmids alone (Fig. 2A, lanes 8 and 11). In each case, the major product mapped to a position one nucleotide beyond the authentic 5′ terminus and represented capped RNA 3, as seen in previous experiments (4). RNA 3 was also detected in cells cotransfected with both plasmids (Fig. 2A, lanes 10 and 13), although with a marked decrease in band intensity, consistent with the inhibition of RNA 3 synthesis mediated by RNA 2 replication (36) (Fig. 1). As expected, RNA 3 was not detected in cells that had been transfected with FHV2 plasmids alone (Fig. 2A, lanes 9 and 12) or in mock-transfected cells (Fig. 2A, lane 7). Primer extension thus confirmed the identity of RNA 3 and thereby provided further evidence for replication of primary FHV1 transcripts in cells transfected with the pol II-FHV1 plasmids.

RNA 2 species were analyzed by extension of an oligonucleotide primer complementary to nt 99 to 81 of FHV RNA 2, together with the 18S rRNA primer. As observed with the RNA 3-specific primer and as seen in previous experiments (3, 6), a family of three primer extension products was detected with each RNA template (Fig. 2B), the major product corresponding to authentic capped RNA 2. This product was detected in cells transfected with RNA isolated from purified FHV virions (Fig. 2B, lane 1) and at a low level on transfection with purified RNA 1 (Fig. 2B, lane 2), indicating a slight contamination of the RNA 1 preparation with RNA 2. As expected, RNA 2 was not detected in cells transfected with either of the FHV1 plasmids alone (Fig. 2B, lanes 4 and 7) or in mock-transfected cells (Fig. 2B, lane 3). Neither were primary transcripts of RNA 2 detected in cells transfected with either of the FHV2 plasmids alone (Fig. 2B, lanes 5 and 8), presumably because their concentrations were below the detection limit of this assay. However, capped RNA 2 molecules were detected when the cells were cotransfected with the FHV1 and FHV2 plasmids (Fig. 2B, lanes 6 and 9). The ability to detect RNA 2 only in the presence of replicating RNA 1 (which provides the RNA replicase in *trans*) indicates that FHV2 primary transcripts were replicating in an RNA 1-dependent reaction, as observed during a natural FHV infection (18, 36).

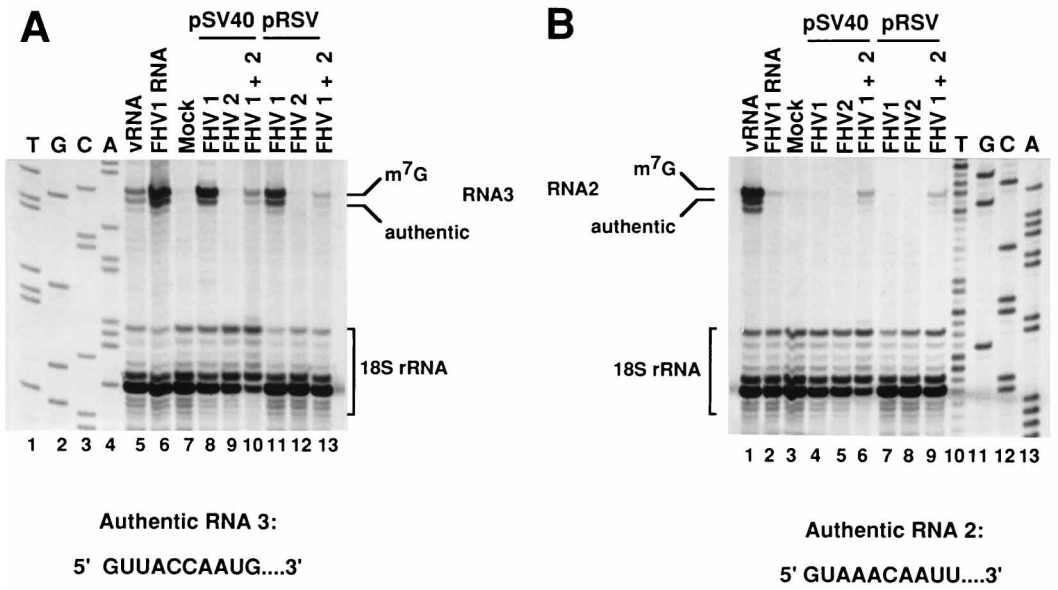

FIG. 2. Primer extension analysis of RNAs isolated from cells transfected with FHV1 and FHV2 plasmids. BHK21 cells were transfected as described for Fig. 1. At 4 days posttransfection, RNAs were isolated and analyzed by primer extension with the mixture of oligonucleotide primers indicated below. The products, labeled by incorporation of [α-^{35}S]dATP, were separated on a 6% acrylamide sequencing gel alongside a dideoxynucleotide sequencing ladder generated with the same FHV-specific primers on pRSV-FHV1[0,0] (A) and pSV40-FHV2[0,0] (B) plasmid DNA. Autoradiographs of the dried gels are shown. vRNA, intracellular RNA from cells transfected with FHV virion RNA; FHV1 RNA, intracellular RNA from cells transfected with purified FHV RNA 1. (A) RNA 3-specific primer (complementary to nt 2819 to 2801 of RNA 1) and 18S rRNA primer (complementary to nt 82 to 65 of *Rattus norvegicus* 18S rRNA) (32). (B) RNA 2-specific primer (complementary to nt 99 to 81 of RNA 2) and 18S rRNA primer. The 5′-terminal sequence of RNA 3 is shown at the bottom of panel A, and that of RNA 2 is shown at the bottom of panel B. The lanes of the sequencing ladder are labeled with the complement of the terminating dideoxynucleotide so the sequence of the positive-sense template can be read directly.

Comparison of three different FHV expression systems. A series of experiments was performed in which we directly compared the pol II system to the two VV-based systems (1, 3, 4, 6, 7). We first examined the rates of synthesis of RNAs 1 and 3 as a function of time after transfection or infection. BHK21 cells either were transfected with pSV40-FHV1[0,0], or were infected with vTF7-3 and transfected with the T7-based plasmid FHV1[1,0] (1), or were infected with the VV-FHV1 recombinant vF1 (7). The cells were incubated at 28°C for various times and then labeled with [^{3}H]uridine in the presence of act D; total cellular RNAs were isolated and analyzed as described for Fig. 1.

Radiolabeled RNAs 1 and 3 were first detected at 3 days posttransfection with pSV40-FHV1[0,0] (Fig. 3, lane 5), and synthesis of both RNAs continued at an increasing rate through 5 days posttransfection (Fig. 3, lanes 6 and 7), when the incubation was terminated. In similar experiments, synthesis of labeled RNAs 1 and 3 continued even 10 days posttransfection, despite the fact that the DNA hybridization signal from the input plasmid diminished to background levels during this time (data not shown). No cytopathology was detected by light microscopy of these transfected cultures.

In cells infected with vTF7-3 and transfected with pT7-FHV1[1,0] (Fig. 3, lanes 9 to 12), labeled RNAs 1 and 3 were synthesized in abundance at 24 h posttransfection, reached a maximal rate of synthesis by 48 h, and exhibited a gradual decline through 4 days posttransfection. In the third experimental situation, cells were infected with recombinant vF1 (Fig. 3, lanes 14 to 18). Labeled RNAs 1 and 3 were first detected at 24 h, at which time they had already reached a maximal rate of synthesis that remained relatively constant through 4 days and then declined by day 5.

These data are consistent with the interpretation that the rates of RNA replication were similar in the three experimental situations on a per-cell basis, but that RNA replication occurred in about 10, 50, and 100% of the cells, respectively. These numbers agree with the estimated 10% efficiency of transfection observed with pSV40-FHV1[0,0] (data not shown), compared with the 50% efficiency of productive transfection of vTF7-3-infected cells (1, 3, 4, 6) and the 100% efficiency of infection with vF1 at multiplicity of infection of 10 (7).

We analyzed the RNAs from the experiment shown in Fig. 3 by primer extension, as described for Fig. 2A, with primers specific for RNA 3 and 18S rRNA. An autoradiograph of the gel was quantitated by densitometry, and the levels of RNA 3 were normalized to the amount of rRNA in each sample. The resulting values were plotted as a function of time of incubation (Fig. 4). As observed by metabolic labeling, RNA 3 was first detected in the VV-based systems 24 h after transfection and/or infection, but the peak accumulation in each case was reached after 48 h. RNA 3 derived from pSV40-FHV1[0,0] was first detected at 3 days posttransfection and increased in abundance up to day 5. Interestingly, however, RNA 3 accumulated significantly more in the pol II-initiated system than would be predicted from its relative rate and duration of synthesis in the VV-based systems (compare Fig. 3 and 4). We attribute this to a less rapid turnover of the FHV RNAs in uninfected cells, as expected from the general labilization of mRNAs that results from infection with VV (28). This was an additional and unforeseen benefit of using cellular transcription to launch RNA replication.

We speculate that the considerable delay in the onset of RNA replication in the pol II system (Fig. 3 and 4) can be attributed to the requirements for transport of DNA and RNA into and out of the nucleus, respectively, before cytoplasmic

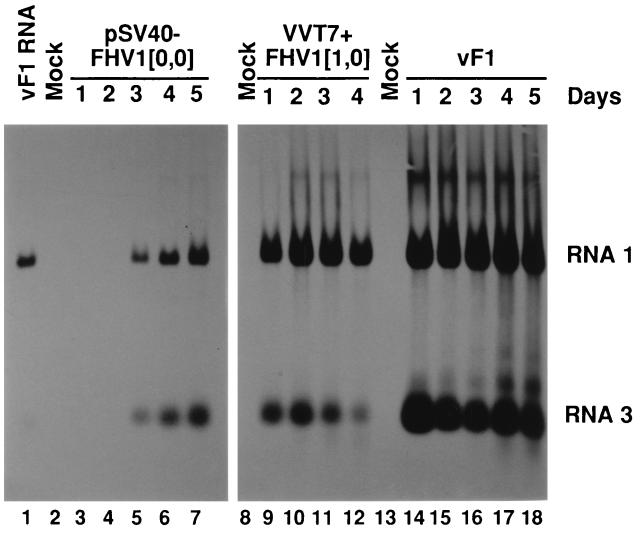

FIG. 3. Comparison of the rates of replication of primary FHV1 transcripts made by pol II, T7 RNA polymerase, and VV RNA polymerase. BHK21 cells in parallel dishes were either transfected with 1.3 μg of pSV40-FHV1[0,0] plasmid DNA (lanes 3 to 7), infected with vTF7-3 at a multiplicity of infection of 10 PFU per cell and transfected with 5 μg of pT7-FHV1[1,0] (lanes 9 to 12), or infected with vF1 at a multiplicity of infection of 10 (lanes 14 to 18) and incubated at 28°C for the times shown. The cells were then labeled for 2 h with [^{3}H]uridine in the presence of act D. Total cellular RNAs were isolated, separated on a formaldehyde-agarose gel, and visualized by fluorography, as described for Fig. 1. vF1, intracellular RNA from cells transfected with vF1-derived RNA.

RNA replication can become established. Transfected plasmids that contain pol II promoters need to gain access to the nucleus to be transcribed by the cellular RNA polymerase. The primary transcripts must then be exported to the cytoplasm before replication can begin, but unlike the vast majority of cellular pol II transcripts (9, 10, 22), they must avoid being spliced during transport. In contrast, in the VV-based systems, primary transcription takes place in the cytoplasm, the same cellular compartment in which RNA replication occurs, and no such delay is observed.

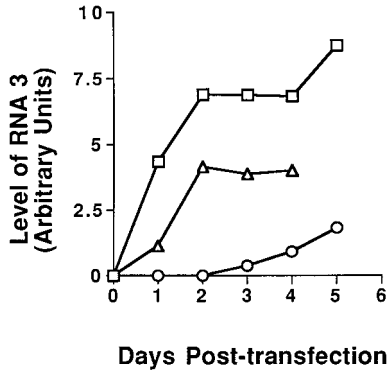

FIG. 4. Comparison of RNA 3 accumulation in the pSV40-FHV1[0,0], vTF7-3/FHV1[1,0], and vF1 systems. BHK21 cells were transfected and/or infected as described for Fig. 3 and incubated at 28°C. Total cellular RNAs were isolated at the times shown, and RNA 3 was quantitated by primer extension with a mixture of two oligonucleotide primers, one complementary to nt 2819 to 2801 of RNA 1 (80 to 98 nt downstream of the 5′ end of RNA 3) and the other complementary to nt 82 to 65 of 18S rRNA. Products were separated on a 6% polyacrylamide sequencing gel. An autoradiograph of the gel was scanned on a DeskTop Plus densitometer (pdi, Huntington Station, N.Y.), the data were analyzed with the Quantity One software package (pdi), and the levels of RNA 3 were normalized to the amount of 18S rRNA in each sample. The resulting values were plotted as a function of incubation time. □, vF1; △, VVT7/T7-FHV1[1,0]; ○, pSV40-FHV1[0,0].

Attempts to accelerate the onset of replication by adding pol II termination and polyadenylation signals and splice junctions to the pol II-based plasmids were unsuccessful (data not shown), perhaps because we were forced to place them downstream of the ribozyme to avoid perturbing the RNA replicon. However, similar delayed kinetics were seen by Herweijer et al. (21) and Dubensky et al. (13) when they transfected cells with Sindbis virus DNA vectors that expressed luciferase from retroviral promoters and that contained termination, polyadenylation, and splicing signals.

These results illustrate the potential of FHV RNA replication and, by extension, nodaviral RNA replication in general as the basis for both transient and long-term expression vectors. The ability to launch RNA replication with a cellular RNA polymerase to make the primary transcripts circumvents our previous dependence on VV and thereby greatly extends the possible applications of this expression system. It now seems feasible to work towards the development of vectors that use nodaviral RNA replication for the controlled, long-term production of abundant amounts of heterologous mRNAs even in quiescent cells.

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