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The Nucleoprotein Is Required for Efficient Coronavirus Genome Replication

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The construction of a set of transmissible gastroenteritis coronavirus (TGEV)-derived replicons as bacterial artificial chromosomes is reported. These replicons were generated by sequential deletion of nonessential genes for virus replication, using a modified TGEV full-length cDNA clone containing unique restriction sites between each pair of consecutive genes. Efficient activity of TGEV replicons was associated with the presence of the nucleoprotein provided either in *cis* or in *trans*. TGEV replicons were functional in several cell lines, including the human cell line 293T, in which no or very low cytopathic effect was observed, and expressed high amounts of heterologous protein.

Since the etiologic pathogen causing severe acute respiratory syndrome was identified as a coronavirus (6, 8, 12, 15, 16, 18, 25), the study of coronavirus molecular biology has acquired significant relevance in order to develop effective strategies to prevent and control coronavirus infections. The design of antiviral drugs interfering with coronavirus replication is a rational approach requiring a detailed study of the replication mechanism at the molecular level. Coronavirus-derived replicons should be useful tools to select effective interfering molecules.

Transmissible gastroenteritis coronavirus (TGEV) is a member of the *Coronaviridae* family within the order *Nidovirales*, composed of enveloped single-stranded, positive-sense RNA viruses relevant in animal and human health (7). About two-thirds of the 28.5-kb TGEV genome encodes the replicase gene, which comprises open reading frames 1a and 1b, the last one being expressed by ribosomal frameshifting (23). Translation of both open reading frames results in the synthesis of two large polyproteins that are processed by viral proteinases to yield the replicase-transcriptase complex (38). The 3′ one-third of the genome includes the genes encoding the structural and nonstructural proteins, in the order 5′-S-3a-3b-E-M-N-7-3′. These proteins are expressed by a discontinuous transcription process that most probably takes place during the synthesis of the negative strand, leading to the generation of a 3′ coterminal nested set of subgenomic mRNAs, each of which has at its 5′ end a capped leader sequence derived from the 5′ end of the genome (26, 39). Synthesis of subgenomic negative-sense RNA species is regulated by the transcription-regulating sequences (TRSs), which include a highly conserved core sequence that is found preceding each gene and at the 3′ end of the leader sequence (3).

Little is known about the genome replication of coronavirus at the molecular level, and until recently, study was restricted to the analysis of defective interfering RNA genomes that are amplified by a helper virus (13, 24, 34), temperature-sensitive mutants that are defective in RNA synthesis (27, 29), and the analysis of recombinant viruses generated by targeted recombination (19). The recent construction of coronavirus full-length cDNA clones (2, 4, 30, 35–37) provides an opportunity for the genetic manipulation of coronavirus genomes to study fundamental viral processes and to develop expression vectors. In fact, the generation of coronavirus self-replicating RNAs (replicons) provides a valuable avenue to explore the molecular bases of coronavirus genome replication. This approach has been used to analyze the replication and transcription mechanism of many positive-strand RNA viruses (1, 11, 17, 20, 31, 33).

In this article, we report the generation and analysis of a collection of TGEV-derived replicons that are functional in several cell lines and express high amounts of heterologous proteins. In addition, our data indicate that the N protein is required either in *cis* or in *trans* for efficient coronavirus-based replicon activity.

Strategy for construction of TGEV-derived replicons. The generation of a TGEV replicon was based in a first approach on published data showing that for many positive-strand RNA viruses, only the replicase gene was needed for autonomous replication of the viral RNA (9, 14, 17, 20). Accordingly, a cDNA that contains the 5′ and 3′ ends of the TGEV genome and the replicase gene was cloned as a bacterial artificial chromosome (BAC) under the control of the cytomegalovirus (CMV) immediate-early promoter following the same approach as that described for the generation of a TGEV full-length cDNA clone (2, 10). Unfortunately, after transfection of swine testis (ST) cells with the replicon, using Lipofectine (Invitrogen), no significative replicase activity was detected (data not shown). One possible explanation for this lack of activity could be the low transfection efficiency of ST cells (less of 0.1%) with large-size coronavirus replicons. To test this possibility, the transfection efficiency of TGEV BAC clones was optimized. Baby hamster kidney cells stably transformed with the gene encoding the porcine aminopeptidase N (BHK-pAPN) (5) and human 293T cells (American Type Culture Collection) were used in the optimization process, as they are

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transfected with efficiencies about 10- and 20-fold higher, respectively, than ST cells and because they also are permissive for TGEV replication. After more than 12 commercial transfection reagents were tested, Lipofectamine 2000 (Invitrogen) was selected for transfection of BACs containing TGEV-derived replicon cDNA clones (about 30 kb), leading to transfection efficiencies of 20 and 42% in BHK-pAPN and 293T cells, respectively (F. Almazán, C. Galán, and L. Enjuanes, unpublished data).

After optimization of BAC transfection, a collection of TGEV replicons was generated to study the roles of other viral genes, in addition to the replicase gene, on coronavirus replicon activity. Genes 3a, 3b, and 7 are nonessential for virus replication in culture cells, and gene E, although essential for virus production, does not affect genome replication (21, 22, 28). A modified TGEV infectious cDNA clone (TGEV-RS), in which overlapping genes were separated by duplication of TRSs and introduction of unique restriction sites at the 5′ end of each gene (22), was used for the construction of three TGEV replicons by sequential deletion of structural and nonstructural genes nonessential for virus genome replication: (i) REP 1, containing the untranslated 5′ and 3′ regions of the genome, the replicase, M, N, and 7 genes; (ii) REP 2, in which gene M was deleted; (iii) and REP 3, with genes M and N deleted (Fig. 1A). Gene 7 was maintained in all cases because it may contain necessary *cis* elements for genome replication due to its proximity to the 3′ end of the TGEV genome. Expression of gene 7 mRNA was used to study replicon functionality by reverse transcription-PCR (RT-PCR) analysis. Additionally, a multicloning site containing unique restriction sites, AvrII, MluI, SwaI, and FseI, was cloned downstream of the replicase gene to allow cloning and expression of heterologous genes. The detailed cloning strategy, plasmid maps, and sequences are available from the authors upon request.

Activity of TGEV-derived replicons in several cell lines. To study the functionality of TGEV replicons, human 293T cells, which are transfected with efficiencies over 40%, were used in the assay. 293T cells were grown to 95% confluence on 35-mm-diameter plates and transfected with 4 μg of either TGEV replicons, the full-length cDNA clone, or a nonreplicative cDNA clone, using 12 μg of Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. Total intracellular RNA was extracted at 1.5, 5, and 10 days posttransfection (dpt) with the RNeasy Mini Kit (QIAGEN) and used as the template for RT-PCR analysis of gene 7 mRNA transcription. RT reactions were performed with Moloney murine leukemia virus reverse transcriptase (Ambion) and the antisense primer 7(213)RS (5′-TCTGTAGCAGCAAAATCC-3′), complementary to nucleotides 199 to 216 of gene 7. The cDNAs generated were amplified by PCR using the reverse primer 7(213)RS and the forward primer SP (5′-GTGAGTGTAGCGTGGCTATA TCTCTTC-3′), spanning nucleotides 15 to 41 of the TGEV leader sequence. High levels of gene 7 mRNA were detected in cells transfected with REP 1 and 2 (Fig. 1B), indicating that both replicons are functional, at least at the transcription level. However, no transcript was detected in cells transfected with REP 3, lacking the N gene, indicating a possible role of N protein in replicon activity (Fig. 1B). Interestingly, gene 7 transcripts were detected in REP 1 and 2 even after 10 dpt (three cell passages), suggesting that these replicons had very little cytopathicity or were noncytopathic in human cells. Identical experiments were performed with BHK-pAPN and ST cells, but in these cases TGEV replicons were cytopathic and gene 7 mRNA was detected only in the samples analyzed up to 36 h posttransfection (hpt) but not later (data not shown). A quantitative analysis of gene 7 mRNA in transfected 293T, BHK-pAPN, and ST cells was performed by real-time RT-PCR. The primers used for RT (7RS, 5′-AAAACTGTAATA AATACAGCATGGAGGAA-3′, complementary to nucleotides 10 to 38 of gene 7) and PCR (reverse primer 7RS and the forward primer LDVS, 5′-CGTGGCTATATCTCTTCTTTTA CTTTAACTAG-3′, spanning nucleotides 24 to 55 of the TGEV leader sequence) were designed by using the Primer Express software (Applied Biosystems). SYBR Green PCR master mix was used in the PCR step following the manufacturer's specifications (Applied Biosystems). In 293T cells the amount of transcript was practically identical in the samples analyzed at 1.5 and 10 dpt, in contrast to those in BHK-pAPN and ST cells, in which transcript expression was undetectable after 2 dpt, reinforcing the idea that TGEV replicons are noncytopathic in human 293T cells. As the TGEV replication level was about threefold lower in 293T cells than in BHK-pAPN cells (data not shown), this could explain the fact that TGEV replicons were cytopathic in BHK-pAPN cells and not in 293T cells. However, replicon toxicity could also be due to a cell-specific factor. Finally, the amount of the cDNA encoding the replicon RNA and the RNA itself were analyzed at different dpt. Replicon RNA showed a higher stability than the cDNA (data not shown), suggesting that the TGEV replicon RNA was also functional at the replication level.

Role of N protein provided either in *cis* or in *trans* in replicon activity. To confirm whether the N protein is important for TGEV replicon activity, the synthesis of gene 7 mRNA was analyzed by RT-PCR in BHK-pAPN cells transformed with the Sindbis virus replicon pSINrep21 (9) expressing TGEV N protein. To show the specificity of N protein expression, similar analyses were carried out with BHK-pAPN cells and BHK-pAPN cells transformed with the Sindbis virus replicon alone or with the replicon expressing the TGEV E protein (Fig. 2A and B). The indicated cells were grown to 95% confluence on 35-mm-diameter plates and transfected with 4 μg of each TGEV replicon, using 12 μg of Lipofectamine 2000 (Invitrogen). At 36 hpt total intracellular RNA was extracted and used as a template for RT-PCR analysis of gene 7 mRNA following the procedure described above. Both, REP 1 and 2 were functional in all analyzed cells. However, in the case of REP 3 high levels of gene 7 mRNA were detected only in cells expressing TGEV N protein. These results indicated that N protein provided either in *cis* or in *trans* is required for efficient replicon activity. A quantitative analysis of gene 7 mRNA in BHK-pAPN cells or BHK-pAPN cells expressing TGEV N protein transfected with TGEV replicons was performed by real-time RT-PCR using the primers 7RS and LDVS, described above (Fig. 2C). A basal activity of REP 3 was detected, but the replicon activity increased more than 100-fold when N protein was provided in *cis* (REP 1 and REP 2 versus REP 3 activity in BHK-pAPN cells) and more than 1,000-fold when N protein was provided in *trans* (REP 3 activity in BHK-pAPN cells expressing TGEV N protein versus REP 3 activity in BHK-

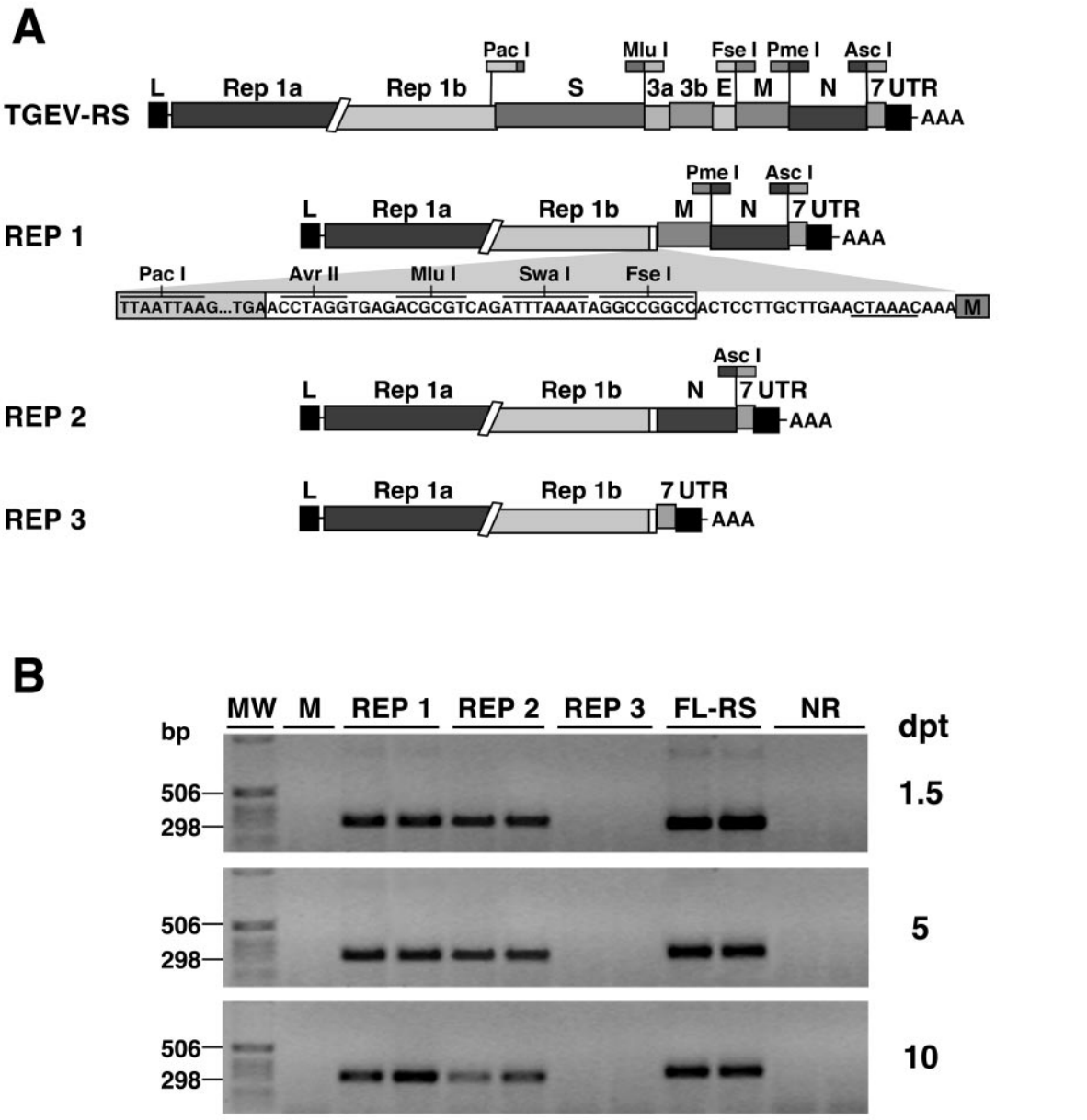

FIG. 1. Functional analysis of TGEV-derived replicons. (A) Genetic structures of TGEV-derived replicons. The genetic structures of the TGEV cDNA clone (TGEV-RS) and the replicons generated from this cDNA (REP 1, REP 2, and REP 3) are illustrated. To construct REP 1, a 125-bp fragment containing the restriction sites PacI, AvrII, MluI, SwaI, and FseI, generated by PCR using two overlapping oligonucleotides, was cloned in TGEV-RS digested with PacI and FseI. REP 2 and REP 3 were generated from REP 1 by deletion of fragments SwaI-PmeI and SwaI-AscI, respectively. To avoid possible interferences with the expression of heterologous genes, the TRS of the S gene, located at the 3′ end of the replicase gene, was eliminated by introduction of three silent point mutations in its conserved core sequence. Letters and numbers above the bars indicate the viral genes. L, leader sequence; UTR, untranslated region. Relevant restriction sites are indicated. The core sequence is underlined. (B) Functional analysis of TGEV-derived replicons by RT-PCR. Human 293T cells were mock transfected (M) or transfected with TGEV replicons (REP 1, 2, and 3), the full-length cDNA clone (FL-RS), or a nonreplicative cDNA clone (NR), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. Total intracellular RNA was isolated at 1.5, 5, and 10 dpt and analyzed by RT-PCR with specific oligonucleotides to detect gene 7 mRNA. Duplicate RT-PCR products amplified in parallel were resolved by electrophoresis in 1% agarose gels. MW, molecular weight markers.

pAPN cells). Interestingly, the activity of REP 1 and 2, carrying the N gene, was also increased when the N protein was provided in *trans*, suggesting that the N protein accumulation level is a critical factor or that the presence of N protein is important at the beginning of the replication process.

To discard the possibility that gene N mRNA itself was responsible for TGEV replicon activity enhancement, the activity of REP 3 was analyzed by real-time RT-PCR in BHK-pAPN cells transformed with the Sindbis virus replicon expressing either gene N mRNA or the same mRNA mutated in order to eliminate N protein synthesis. To achieve this objective, three different mutants were constructed, in which one,

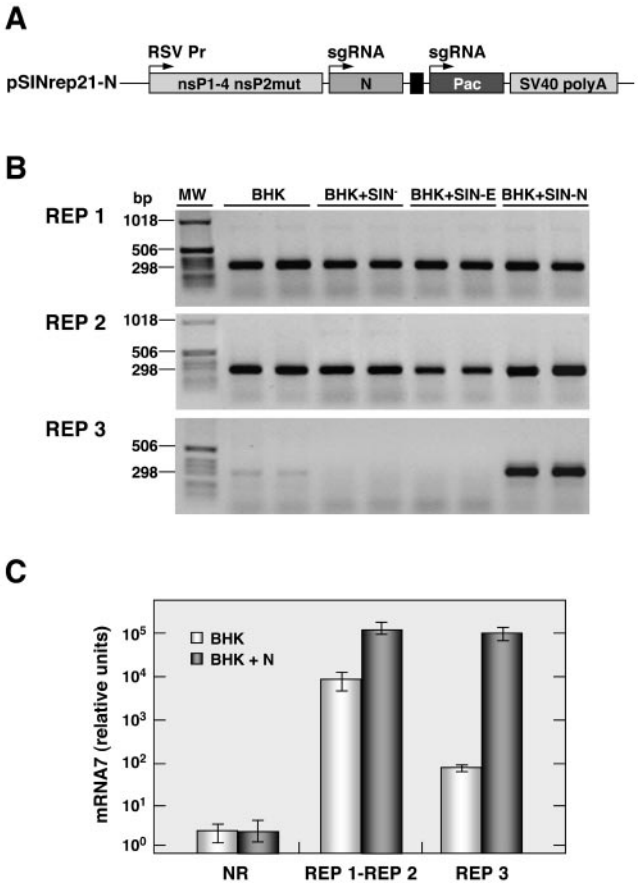

FIG. 2. Effect of N protein on TGEV-derived replicon activity. (A) Scheme of the Sindbis virus replicon construct encoding the TGEV N gene. RSV Pr, Rous sarcoma virus promoter; sgRNA, subgenomic RNA; nsP1-4, nonstructural proteins 1 to 4; nsP2mut, mutant of nonstructural protein 2; Pac, puromycin resistance gene; SV40 poly A, transcription-termination polyadenylation signal from simian virus 40. (B) RT-PCR analysis of gene 7 mRNA. BHK-pAPN cells (BHK) or BHK-pAPN cells transformed with either the Sindbis virus replicon pSINrep21 alone (BHK + SIN^-) or pSINrep21 expressing TGEV E protein (BHK + SIN-E) or TGEV N protein (BHK + SIN-N) were transfected with TGEV replicons (REP 1, 2, and 3), using Lipofectamine 2000 (Invitrogen) following the manufacturer's specifications. At 36 hpt total intracellular RNA was extracted and used as the template for RT-PCR analysis with specific primers to detect gene 7 mRNA. Duplicate RT-PCR products amplified in parallel were resolved by electrophoresis in 1% agarose gels. MW, molecular weight markers. (C) Real-time RT-PCR quantification of gene 7 mRNA. The amount of mRNA7, expressed as relative units, was determined by real-time RT-PCR with specific oligonucleotides to detect gene 7 mRNA in RNA samples isolated at 36 hpt from BHK-pAPN cells (BHK) or BHK-pAPN cells expressing N protein (BHK + N) transfected with either a nonreplicative cDNA clone (NR), REP 1, REP 2, or REP 3. REP 1-REP 2 indicates mean values from REP 1 and 2, both encoding the N gene. Mean values from three experiments are represented, with standard deviations shown as error bars.

two, or three ATGs in the N gene coding sequence were mutated to ATC (Fig. 3A). Expression of gene 7 mRNA was used to study replicon activity following the procedure described above. The synthesis of gene N mRNA by the Sindbis virus replicon was analyzed by real-time RT-PCR using the antisense primer 5′-CCAATAACCAATCTGTTGATCCCT-3′, complementary to nucleotides 187 to 219 of the N gene, and the forward primer 5′-ACTTATGCTCGAGAGACTTTGTA CCC-3′, spanning nucleotides 146 to 171 of the N gene. A clear reduction in the activity of REP 3 (more than 85%) was detected in BHK-pAPN cells expressing the different gene N mRNA mutants. However, no differences in the expression of gene N mRNA between the wild type and the different mutants were detected, indicating that N protein itself and not its mRNA was responsible for TGEV replicon enhancement (Fig. 3B).

These data indicated that N protein is not strictly essential, but it plays an important role as an enhancer of coronavirus replicon activity. The mechanism mediating N protein enhancement activity is unknown. N protein may be a critical component of the replication-transcription complex, or it may simply play a structural role to stabilize coronavirus replicon RNAs. This is the first time that a detailed and quantitative analysis of the requirement for N protein in coronavirus replication or transcription has been reported, although it was previously observed that N protein increases the rescue

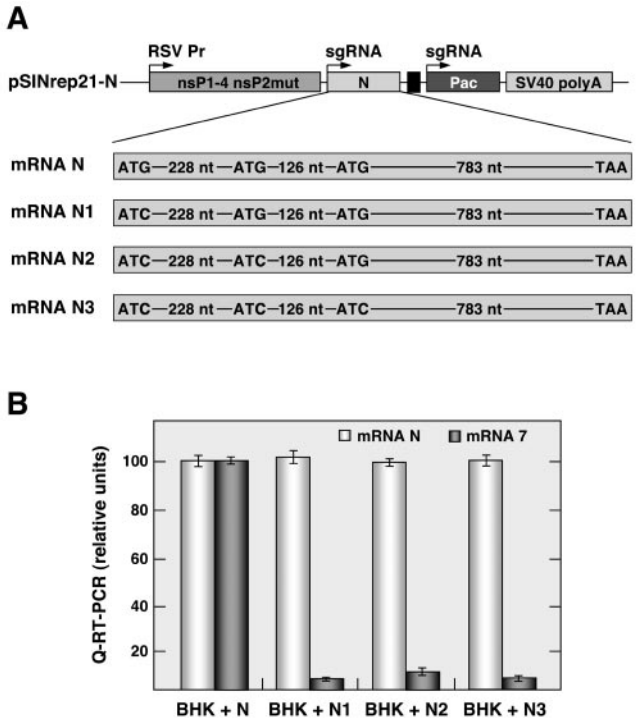

FIG. 3. Effect of N protein and its mRNA on TGEV-derived replicon activity. (A) Schematic representation of the Sindbis virus replicon encoding the different gene N mRNA mutants. The positions of the three ATGs in the N gene coding sequence, as well as the point mutations introduced, are indicated. The designations above the top bar are the same that those described for Fig. 2A. (B) Real-time RT-PCR quantification of genes 7 and N mRNAs. The amounts of genes 7 and N mRNAs, expressed as relative units, were determined by real-time RT-PCR with specific oligonucleotides in RNA samples isolates at 36 hpt from BHK cells expressing the wild-type gene N mRNA (BHK + N) or gene N mRNA with the first ATG mutated (BHK + N1), with the two first ATGs mutated (BHK + N2), or with the three ATGs mutated (BHK + N3) transfected with REP 3. Mean values from two experiments are represented, with standard deviations shown as error bars.

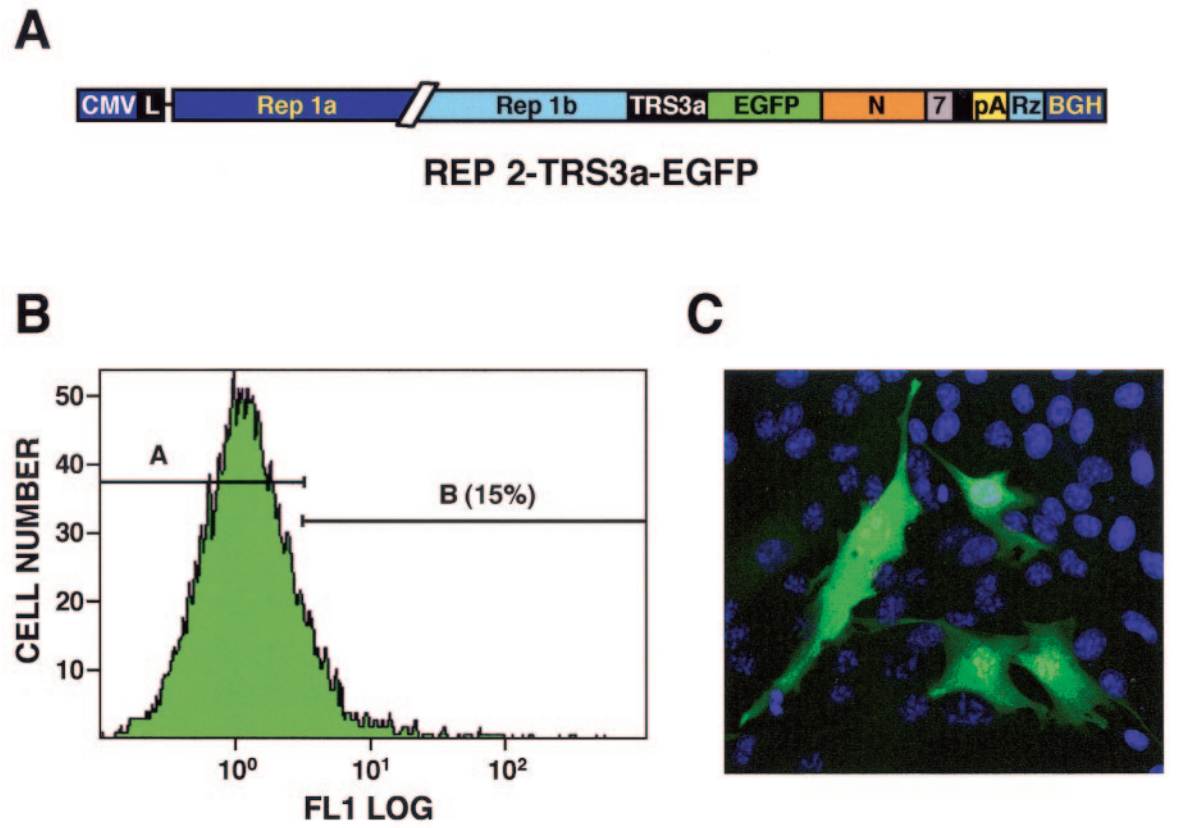

FIG. 4. Analysis of EGFP expression by TGEV-derived REP 2. (A) Schematic structure of the cDNA encoding the REP2-TRS3a-EGFP RNA with the EGFP gene under the control of gene 3a TRS. The numbers and letters inside the boxes indicate the viral genes. L, leader sequence; CMV, cytomegalovirus immediate-early promoter; pA, poly(A); Rz, hepatitis delta virus ribozyme; BGH, bovine growth hormone termination and polyadenylation sequences. (B) Flow cytometry analysis of EGFP expression in BHK-pAPN cells transfected with REP2-TRS3a-EGFP. A and B represent the EGFP-negative and -positive cell populations, respectively. Green fluorescence intensity is revealed in logarithmic units on the *x* axis. (C) EGFP expression analyzed by confocal microscopy of BHK-pAPN cells transfected with REP2-TRS3a-EGFP. Cell nuclei were stained with TOPRO 3 (Molecular Probes).

of coronavirus from infectious RNA transcripts (4, 32, 35, 37).

Heterologous gene expression from TGEV-derived replicons. In order to evaluate the potential of TGEV replicons to express heterologous genes, the expression of enhanced green fluorescent protein (EGFP) by REP 2 was analyzed in BHK-pAPN cells. A 1,115-bp cDNA fragment containing the EGFP gene downstream of native gene 3a TRS, comprising 262 nucleotides of the 5′ TRS, the core sequence, and 23 nucleotides of the 3′ TRS preceding the translation initiation codon, was amplified by PCR with the forward primer 5′-GCGGATATCTGTTTAAACGTGTGGCTACTAATAGGCTTAGTAG-3′ (the restriction sites EcoRV and PmeI are underlined) and the reverse primer 5′-GGGGATATCGGCGCCAATATTTAAATAAAATCACCATTGAG-3′ (the restriction sites EcoRV and SfoI are underlined), using as the template the plasmid pBAC-TGEV-Δ3ab-EGFP (a full-length TGEV cDNA clone expressing EGFP under the control of TRS3a). The PCR product (TRS3a-EGFP) was digested with restriction endonuclease EcoRV and cloned into REP 2 digested with MluI and treated with Klenow enzyme to generate the plasmid REP2-TRS3a-EGFP (Fig. 4A). BHK-pAPN cells were grown to 95% confluence and transfected with REP2-TRS3a-EGFP, using Lipofectamine 2000 (Invitrogen) as described in the previous section, and EGFP expression was analyzed at 36 hpt by flow cytometry (Fig. 4B) and confocal microscopy (Fig. 4C). A significative proportion of cells (18%) were transfected, and more than 80% of them expressed high levels of EGFP (Fig. 4C), indicating that a majority of cells transfected with the replicon expressed the heterologous gene. The strategy described here, in which the replicon encoding the N protein was launched from the cell nucleus by using the CMV promoter, allowed heterologous protein expression levels at least 1,000-fold higher than those previously reported with the human coronavirus 229E replicon (31).

The availability of functional replicons will significantly facilitate the study of the coronavirus replication mechanism and the production of safe TGEV-derived vectors for vaccination and possibly gene therapy.

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REFERENCES

1. **Agapov, E. V., I. Frolov, B. D. Lindenbach, B. M. Pragai, S. Schlesinger, and C. M. Rice.** 1998. Noncytopathic Sindbis virus RNA vectors for heterologous gene expression. Proc. Natl. Acad. Sci. USA **95:**12989–12994.
2. **Almazán, F., J. M. González, Z. Pénzes, A. Izeta, E. Calvo, J. Plana-Durán, and L. Enjuanes.** 2000. Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. Proc. Natl. Acad. Sci. USA **97:** 5516–5521.
3. **Alonso, S., A. Izeta, I. Sola, and L. Enjuanes.** 2002. Transcription regulatory sequences and mRNA expression levels in the coronavirus transmissible gastroenteritis virus. J. Virol. **76:**1293–1308.
4. **Casais, R., V. Thiel, S. G. Siddell, D. Cavanagh, and P. Britton.** 2001. Reverse genetics system for the avian coronavirus infectious bronchitis virus. J. Virol. **75:**12359–12369.
5. **Delmas, B., J. Gelfi, H. Sjöström, O. Noren, and H. Laude.** 1994. Further characterization of aminopeptidase-N as a receptor for coronaviruses. Adv. Exp. Med. Biol. **342:**293–298.
6. **Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz, and H. W. Doerr.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1967–1976.
7. **Enjuanes, L., W. Spaan, E. Snijder, and D. Cavanagh.** 2000. Nidovirales, p. 827–834. *In* M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carsten, M. K. Estes, S. M. Lemon, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.), Virus taxonomy. Academic Press, New York, N.Y.
8. **Fouchier, R. A., T. Kuiken, M. Schutten, G. van Amerongen, G. J. van Doornum, B. G. van den Hoogen, M. Peiris, W. Lim, K. Stohr, and A. D. Osterhaus.** 2003. Aetiology: Koch's postulates fulfilled for SARS virus. Nature **423:**240.
9. **Frolov, I., T. A. Hoffman, B. M. Pragai, S. A. Dryga, H. V. Huang, S. Schlesinger, and C. M. Rice.** 1996. Alphavirus-based expression vectors: strategies and applications. Proc. Natl. Acad. Sci. USA **93:**11371–11377.
10. **González, J. M., Z. Pénzes, F. Almazán, E. Calvo, and L. Enjuanes.** 2002. Stabilization of a full-length infectious cDNA clone of transmissible gastroenteritis coronavirus by insertion of an intron. J. Virol. **76:**4655–4661.
11. **Herold, J., and R. Andino.** 2001. Poliovirus RNA replication requires genome circularization through a protein-protein bridge. Mol. Cell **7:**581–591.
12. **Holmes, K. V., and L. Enjuanes.** 2003. Virology. The SARS coronavirus: a postgenomic era. Science **300:**1377–1378.
13. **Izeta, A., C. Smerdou, S. Alonso, Z. Pénzes, A. Méndez, J. Plana-Durán, and L. Enjuanes.** 1999. Replication and packaging of transmissible gastroenteritis coronavirus-derived synthetic minigenomes. J. Virol. **73:**1535–1545.
14. **Khromykh, A. A., and E. G. Westaway.** 1997. Subgenomic replicons of the flavivirus Kunjin: construction and applications. J. Virol. **71:**1497–1505.
15. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1953–1966.
16. **Kuiken, T., R. A. Fouchier, M. Schutten, G. F. Rimmelzwaan, G. van Amerongen, D. van Riel, J. D. Laman, T. de Jong, G. van Doornum, W. Lim, A. E. Ling, P. K. Chan, J. S. Tam, M. C. Zambon, R. Gopal, C. Drosten, S. van der Werf, N. Escriou, J. C. Manuguerra, K. Stohr, J. S. Peiris, and A. D. Osterhaus.** 2003. Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. Lancet **362:**263–270.
17. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285:**110–113.
18. **Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton, and R. L. Roper.** 2003. The genome sequence of the SARS-associated coronavirus. Science **300:**1399–1404.
19. **Masters, P. S.** 1999. Reverse genetics of the largest RNA viruses. Adv. Virus Res. **53:**245–264.
20. **Molenkamp, R., H. van Tol, B. C. Rozier, Y. van der Meer, W. J. Spaan, and E. J. Snijder.** 2000. The arterivirus replicase is the only viral protein required for genome replication and subgenomic mRNA transcription. J. Gen. Virol. **81:**2491–2496.
21. **Ortego, J., D. Escors, H. Laude, and L. Enjuanes.** 2002. Generation of a replication-competent, propagation-deficient virus vector based on the transmissible gastroenteritis coronavirus genome. J. Virol. **76:**11518–11529.
22. **Ortego, J., I. Sola, F. Almazán, J. E. Ceriani, C. Riquelme, M. Balasch, J. Plana, and L. Enjuanes.** 2003. Transmissible gastroenteritis coronavirus gene 7 is not essential but influences *in vivo* virus replication and virulence. Virology **308:**13–22.
23. **Pénzes, Z., J. M. González, E. Calvo, A. Izeta, C. Smerdou, A. Méndez, C. M. Sánchez, I. Sola, F. Almazán, and L. Enjuanes.** 2001. Complete genome sequence of transmissible gastroenteritis coronavirus PUR46-MAD clone and evolution of the purdue virus cluster. Virus Genes **23:**105–118.
24. **Repass, J. F., and S. Makino.** 1998. Importance of the positive-strand RNA secondary structure of a murine coronavirus defective interfering RNA internal replication signal in positive-strand RNA synthesis. J. Virol. **72:**7926–7933.
25. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and W. J. Bellini.** 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300:**1394–1399.
26. **Sawicki, S. G., and D. L. Sawicki.** 1998. A new model for coronavirus transcription. Adv. Exp. Med. Biol. **440:**215–219.
27. **Siddell, S., D. Sawicki, Y. Meyer, V. Thiel, and S. Sawicki.** 2001. Identification of the mutations responsible for the phenotype of three MHV RNA-negative ts mutants. Adv. Exp. Med. Biol. **494:**453–458.
28. **Sola, I., S. Alonso, S. Zúñiga, M. Balasch, J. Plana-Durán, and L. Enjuanes.** 2003. Engineering the transmissible gastroenteritis virus genome as an expression vector inducing lactogenic immunity. J. Virol. **77:**4357–4369.
29. **Stalcup, R. P., R. S. Baric, and J. L. Leibowitz.** 1998. Genetic complementation among three panels of mouse hepatitis virus gene 1 mutants. Virology **241:**112–121.
30. **Thiel, V., J. Herold, B. Schelle, and S. G. Siddell.** 2001. Infectious RNA transcribed *in vitro* from a cDNA copy of the human coronavirus genome cloned in vaccinia virus. J. Gen. Virol. **82:**1273–1281.
31. **Thiel, V., J. Herold, B. Schelle, and S. G. Siddell.** 2001. Viral replicase gene products suffice for coronavirus discontinuous transcription. J. Virol. **75:** 6676–6681.
32. **Thiel, V., N. Karl, B. Schelle, P. Disterer, I. Klagge, and S. G. Siddell.** 2003. Multigene RNA vector based on coronavirus transcription. J. Virol. **77:**9790–9798.
33. **Tijms, M. A., L. C. van Dinten, A. E. Gorbalenya, and E. J. Snijder.** 2001. A zinc finger-containing papain-like protease couples subgenomic mRNA synthesis to genome translation in a positive-stranded RNA virus. Proc. Natl. Acad. Sci. USA **98:**1889–1894.
34. **Williams, G. D., R. Y. Chang, and D. A. Brian.** 1999. A phylogenetically conserved hairpin-type 3′ untranslated region pseudoknot functions in coronavirus RNA replication. J. Virol. **73:**8349–8355.
35. **Yount, B., K. M. Curtis, and R. S. Baric.** 2000. Strategy for systematic assembly of large RNA and DNA genomes: transmissible gastroenteritis virus model. J. Virol. **74:**10600–10611.
36. **Yount, B., K. M. Curtis, E. A. Fritz, L. E. Hensley, P. B. Jahrling, E. Prentice, M. R. Denison, T. W. Geisbert, and R. S. Baric.** 2003. Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus. Proc. Natl. Acad. Sci. USA **100:**12995–13000.
37. **Yount, B., M. R. Denison, S. R. Weiss, and R. S. Baric.** 2002. Systematic assembly of a full-length infectious cDNA of mouse hepatitis virus strain A59. J. Virol. **76:**11065–11078.
38. **Ziebuhr, J., E. J. Snijder, and A. E. Gorbalenya.** 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J. Gen. Virol. **81:**853–879.
39. **Zúñiga, S., I. Sola, S. Alonso, and L. Enjuanes.** 2004. Sequence motifs involved in the regulation of discontinuous coronavirus subgenomic RNA synthesis. J. Virol. **78:**980–994.