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Investigation of the Control of Coronavirus Subgenomic mRNA Transcription by Using T7-Generated Negative-Sense RNA Transcripts

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The subgenomic mRNAs of the coronavirus transmissible gastroenteritis virus (TGEV) are not produced in equimolar amounts. We have developed a reporter gene system to investigate the control of this differential subgenomic mRNA synthesis. Transcription of mRNAs by the TGEV polymerase was obtained from negative-sense RNA templates generated in situ from DNA containing a T7 promoter. A series of gene cassettes was produced; these cassettes comprised the reporter chloramphenicol acetyltransferase (CAT) gene downstream of transcription-associated sequences (TASs) (also referred to as intergenic sequences and promoters) believed to be involved in the synthesis of TGEV subgenomic mRNAs 6 and 7. The gene cassettes were designed so that negative-sense RNA copies of the CAT gene with sequences complementary to the TGEV TASs, or modified versions, at the 3′ end would be synthesized in situ by T7 RNA polymerase. Using this system, we have demonstrated that CAT was expressed from mRNAs derived from the T7-generated negative-sense RNA transcripts only in TGEV-infected cells and only from transcripts possessing a TGEV negative-sense TAS. Analysis of the CAT mRNAs showed the presence of the TGEV leader RNA sequence at the 5′ end, in keeping with observations that all coronavirus mRNAs have a 5′ leader sequence corresponding to the 5′ end of the genomic RNA. Our results indicated that the CAT mRNAs were transcribed from the in situ-synthesized negative-sense RNA templates without the requirement of TGEV genomic 5′ or 3′ sequences on the T7-generated negative-sense transcripts (3′-TAS-CAT-5′). Modification of the TGEV TASs indicated (i) that the degree of potential base pairing between the 3′ end of the leader RNA and the TGEV negative-sense TAS was not the sole determinant of the amount of subgenomic mRNA transcribed and (ii) that other factors, including nucleotides flanking the TAS, are involved in the regulation of transcription of TGEV subgenomic mRNAs.

Transmissible gastroenteritis virus (TGEV), a member of the genus *Coronavirus* in the family *Coronaviridae*, is an enveloped virus with a single-stranded, positive-sense RNA genome of approximately 28,000 nucleotides (nt) that is 5′ capped and 3′ polyadenylated. At least six virus-specific subgenomic mRNA species are produced in TGEV-infected cells and form a 3′-coterminal nested set (13, 27, 40); i.e., they have identical 3′ ends but extend for different lengths in the 5′ direction. Most coronavirus genes, on the genomic RNA, are preceded by a nontranslated region of varying length containing a conserved sequence, which has been given the term intergenic sequence (21). On the negative-stranded copy of the genomic RNA, nucleotides within these conserved sequences have been postulated to be involved in the transcription of the subgenomic mRNAs. However, these conserved sequences have been found to reside within some coronavirus genes; e.g., the conserved sequences for TGEV gene 4, infectious bronchitis virus gene M, and mouse hepatitis virus (MHV) gene 5 are found within the preceding open reading frames ORF-3b, ORF-3c, and ORF-4, respectively. Therefore, in this paper we refer to these conserved sequences, on the genomic strand, as transcription-associated sequences (TASs) (9). The lengths of the potential TGEV TASs are variable, ranging from CUAA AC (mRNA 4) to CGAACUAAAC (mRNA 7).

Coronavirus subgenomic mRNAs all possess a 5′ leader sequence which varies in length (60 to 90 nt) depending on the coronavirus. However, the leader sequence is found only at the very 5′ end on coronavirus genomic RNA, implying that synthesis of the subgenomic mRNAs involves fusion of noncontiguous sequences (23, 38) via a discontinuous transcription mechanism (21, 37). The mechanism of coronavirus transcription has not been elucidated. The leader-priming hypothesis postulates that a *trans*-acting leader RNA, transcribed from the 3′ end of a negative copy of the genomic RNA, binds to conserved regions (complementary TASs) on negative-stranded genomic templates and is extended to produce the subgenomic mRNAs (for reviews, see references 20 and 22). In addition to the subgenomic mRNAs, negative-stranded counterparts, whose precise role in the replication cycle of coronaviruses still remains to be elucidated, have been detected in coronavirus-infected cells (31, 34, 35). They have been proposed to serve as templates for the amplification of corresponding or smaller subgenomic mRNAs (2, 32, 32a, 35), whereas others believe that they may be the end products of the transcription and/or replication process (14). An alternative model for the transcription of coronavirus mRNAs in which the TASs act as transcription termination sequences has been proposed by Sawicki and Sawicki (32). In this model negative-stranded RNAs produced from genomic RNA terminate at the TASs and are postulated to hybridize to the TAS at the 3′ end of the leader sequence on the genomic RNA. This would be followed by extension of the nascent negative-

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stranded RNA to produce a negative-sense leader sequence. The resulting negative-stranded RNAs would then be copied into their positive-stranded counterparts, i.e., mRNAs. Both models require a discontinuous transcription step and require an interaction between the TASs and the leader RNA (22). It is possible that both models are involved in the transcription of coronavirus mRNAs, i.e., that leader priming occurs early in infection and negative-sense RNAs become the more predominant templates later in infection (32a).

The amounts of MHV subgenomic mRNAs produced during replication follow a gradational relationship in which shorter mRNAs are produced in larger amounts than longer species. A number of mechanisms have been proposed to account for this observation, including differential premature termination of transcription (16) and the degree of base pairing between the TAS and the leader RNA (4, 36). However, in the TGEV group of coronaviruses this gradational pattern is not observed; the smallest mRNAs of TGEV (13, 27, 40), porcine respiratory coronavirus (28), feline infectious peritonitis virus (6), and canine coronavirus (11) are produced in smaller amounts than the next larger mRNA. In TGEV each TAS contains the sequence ACUAAAC (except for mRNA 4, which contains the sequence CUAAAC), and the extent of potential base pairing between the TAS and the leader RNA varies, which has been proposed to regulate subgenomic mRNA transcription in coronaviruses (4, 36).

In order to investigate the potential interactions between the TGEV TAS and leader RNA, we designed a system in which negative-sense RNA was generated in TGEV-infected cells. The rationale of the system was based on the fact that the two main models postulated for coronavirus transcription rely on negative-sense RNA either as a template, as in the leader-priming model, or for the addition of a negative-sense leader RNA sequence, as in the termination-extension model. We produced a series of gene cassettes in which chloramphenicol acetyltransferase (CAT) protein expression was under the control of the TGEV mRNA 6 and 7 TASs, the only coronavirus-derived sequences. This involved the examination of whether a reporter gene could be expressed from a negative-sense RNA via a TGEV mRNA TAS in the presence of helper virus and whether modification of a TAS or flanking nucleotides could up or down regulate transcription of a CAT mRNA. The system therefore allowed us to investigate the role of the TGEV TASs and flanking sequences in the synthesis of TGEV-directed CAT mRNAs independent of the proposed mechanisms for coronavirus mRNA transcription. Our results provided evidence that potential base pairing between the leader RNA and the TAS is not the sole determinant of mRNA abundance. This supports the recent findings of van der Most et al. (39) that argued against the model proposed by Shieh et al. (36). Furthermore, our data indicate that a TAS is sufficient for transcription of an mRNA from a negative-sense (3′-TAS-CAT-5′) template, which does not contain TGEV genomic 5′ or 3′ sequences, in the presence of helper virus.

MATERIALS AND METHODS

Virus and cells. TGEV, strain FS772/70, was grown in LLC-PK_1 cells in trypsin-containing Eagle minimum essential medium (EMEM) (ICN Flow) (9). The recombinant vaccinia virus vTF7-3 (7) was grown in HTK^- cells in medium containing Eagle minimum essential medium, 0.14% sodium bicarbonate, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 100 U of penicillin per ml, 100 μg of streptomycin per ml, 25 U of mycostatin per ml, and 10% fetal calf serum. The vaccinia virus was partially purified through sucrose as described previously (29). Both viruses were titrated by plaque assay.

Oligonucleotides. Oligonucleotides (Table 1) were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer and purified by either gel electrophoresis (30) or COP cartridges (Cruachem).

Construction of reporter genes. Various modified TGEV TASs (Table 1) were fused to the CAT gene, by PCR with the pCAT Basic Vector (Promega) as the template, to produce a series of gene cassettes, as outlined in Fig. 1. PCR amplifications were carried out with 2.5 U of *Taq* polymerase (Promega), 5 ng of pCAT, and 50 pmol of a TAS oligonucleotide and oligonucleotide 8A3G for 25 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min in an Omnigene thermal cycler (Hybaid Ltd.). A final PCR elongation step was excluded to reduce the addition of an extra 3′ adenine residue to the PCR products, resulting in 3′ overhangs (12), which are reported to produce extraneous T7 RNA polymerase-generated transcripts (33). The PCR-generated gene cassettes, named after the TAS-containing oligonucleotides used for their construction, were sequenced at the 5′ ends by using 7-deaza-GTP, oligonucleotide CP3, and the Sequenase PCR product sequencing kit (U.S. Biochemical/Amersham).

Virus infection and transfection protocol. LLC-PK_1 cells were grown to 90% confluence in 25-cm^2 tissue culture flasks (Falcon) and infected with TGEV at a multiplicity of infection of 10 for 1 h at 37°C. The cells were washed and incubated for 1 h in fresh trypsin medium, infected with vTF7-3 at a multiplicity of infection of 10 in medium with 10% fetal calf serum, and incubated for 2 h. The cells were washed, incubated for a further 2 h in fresh medium, transfected with the PCR products for 5 h, and incubated in fresh medium for 18 h. The transfected PCR products had been prepared by adding 10 μg of the appropriate PCR product to 100 μg of Lipofectamine (Gibco/BRL) in 3 ml of Opti-MEM (Gibco/BRL) for 30 min at room temperature. To account for potential variations in CAT synthesis, duplicate cell sheets were used in two experiments and triplicate cell sheets were used in two experiments.

Preparation of cell extracts. Transfected cells were incubated with ice-cold Tris-EDTA-NaCl buffer (40 mM Tris-HCl [pH 7.8], 1 mM EDTA, 150 mM NaCl) for 5 min, scraped from the flasks, pelleted by centrifugation, washed, and resuspended in 200 μl of CAT enzyme-linked immunosorbent assay (ELISA) sample buffer (Boehringer Mannheim). Five-microliter aliquots were removed for RNA analysis.

Detection and quantification of CAT protein. CAT protein analysis of cell extracts was carried out by CAT ELISA (Boehringer Mannheim) with an initial incubation time of 2 h. The amounts of CAT protein were determined with a Titertek Multiscan MCC/340 MK11 spectrophotometer.

Analysis of intracellular RNA by RT-PCR. Reverse transcription PCR (RT-PCR) analysis was used to determine whether the resultant CAT mRNAs were "coronavirus-like," i.e., whether they possessed a leader RNA sequence. Total cellular RNA was isolated from transfected-cell samples by the method of Chomczynski and Sacchi (5). First-strand cDNA synthesis was carried out with 1 μg of RNA, 500 ng of oligonucleotide CP2 (complementary to a sequence within the CAT gene), and 200 U of Superscript reverse transcriptase (Gibco/BRL) in the presence of 30 U of RNasin. PCR amplifications were carried out with 5 μl of cDNA mix and 50 pmol each of oligonucleotides CP2 and JAHL (corresponding to the 5′ end of the TGEV leader sequence) under the same conditions as described above but with a final elongation step at 72°C for 10 min.

Sequencing of PCR products derived from CAT mRNAs. PCR products were sequenced by using oligonucleotide CP3 (complementary to a sequence within the 5′ end of the CAT gene) either as described above for the construction of reporter genes or from plasmid DNA following cloning into the pGEM-T Vector System (Promega).

In vitro transcription and translation. RNA was synthesized from PCR-generated gene cassettes by using 20 U of T7 RNA polymerase (Promega), 15 nmol each of ATP, UTP, CTP, and GTP, and 2 μg of template DNA at 37°C for 2 h. Template DNA was removed by using 5 U of RQ1 RNase-free DNase (Promega) at 37°C for 30 min, and the RNA was extracted with phenol-chloroform-isoamyl alcohol, precipitated, and redissolved in H_2O to 1 μg/μl. RNA (1 μg) was translated by using 35 μl of nuclease-treated rabbit reticulocyte lysate (Promega), 30 U of RNasin (Promega), and 1 μl of a 1 mM amino acid mixture (Promega) in a total volume of 50 μl at 30°C for 60 min.

RESULTS

Transcription of reporter gene mRNA from negative-sense T7 RNA transcripts via TGEV mRNA 6 and 7 TASs. A reporter gene system was designed to investigate whether a functional mRNA could be produced from a T7-generated negative-sense RNA by the TGEV polymerase. Gene cassettes were produced with the reporter gene (the CAT gene) downstream of potential TGEV TASs followed by a short poly(A) tail and a copy of the T7 promoter (Fig. 1). The cassettes were designed so that negative-sense RNAs of the CAT gene, with a negative copy of a TGEV TAS at the 3′ end, were synthesized by T7 RNA polymerase produced by vTF7-3. Both the leader-priming and termination-extension models, proposed for the transcription of coronavirus mRNAs, predict that if the TGEV polymerase recognizes the T7-generated negative-sense RNA templates, it should result in the synthesis of pos-

TABLE 1. Oligonucleotides used to construct reporter gene cassettes

Oligonucleotide	Sequence[a]	Polarity	Purpose
E6CAT	[ggcctctag]**ACATATGGTATAACTAAACTTCTAA***ATG*GAGAAAAAAATCAC	+	Gene cassette
6JAH4	[ggcctctag]**ACATATGGTATAACTAAACgag***ATG*GAGAAAAAAATCAC	+	Gene cassette
6JAH5	[ggcctctag]**ACATATGGTATCGAACTAAACTTCTAA***ATG*GAGAAAAAAATCAC	+	Gene cassette
6JAH6	[ggcctctag]**ACATATGGTATttCTAAACTTCTAA***ATG*GAGAAAAAAATCAC	+	Gene cassette
6CAT	[ggcctctaga]**GGTATAACTAAACTTCTAA***ATG*GAGAAAAAAATCAC	+	Gene cassette
Sh6CAT	[ggcctctaga]**TATAACTAAACTTCTAA***ATG*GAGAAAAAAATCAC	+	Gene cassette
Sh6JAH4	[ggcctctaga]**TATAACTAAACgag***ATG*GAGAAAAAAATCAC	+	Gene cassette
Sh6JAH5	[ggcctctaga]**TATCGAACTAAACTTCTAA***ATG*GAGAAAAAAATCAC	+	Gene cassette
Sh6JAH6	[ggcctctaga]**TATttCTAAACTTCTAA***ATG*GAGAAAAAAATCAC	+	Gene cassette
S6CAT	[ggcctctag]**ACATATGGTATttagctgaTTCTAA***ATG*GAGAAAAAAATCAC	+	Gene cassette
m6T7	attaatacgactcactataggg**AACTAAACTTCTAA***ATG*GAGAAAAAAATCACTGG	+	Gene cassette
7CAT	[ggcctctaga]**TAACGAACTAAACGAG***ATG*GAGAAAAAAATCAC	+	Gene cassette
7JAH1	[ggcctctaga]**TAAtcAACTAAACGAG***ATG*GAGAAAAAAATCAC	+	Gene cassette
7JAH3	[ggcctctaga]**TAACGAACTAAACttctaa***ATG*GAGAAAAAAATCAC	+	Gene cassette
E7CAT	[ggcctctaga]**TGATGAGGTAACGAACTAAACGAG***ATG*GAGAAAAAAATCAC	+	Gene cassette
S7CAT	[ggcctctaga]**TAAtcgtaccgttGAG***ATG*GAGAAAAAAATCAC	+	Gene cassette
m7T7	attaatacgactcactataggg**AACTAAACGAG***ATG*GAGAAAAAAATCACTGG	+	Gene cassette
8A3G	ccggagatctttattaatacgactcactataggggTTTTTTTT*TTACGCCCCGCCCTGCCACT*	−	Gene cassette
CAT2	TTTTTTTTTTTTTTTTTTTTTTTTTT*TTACGCCCCGCCCTGCCACTCA*	−	Gene cassette
CP2	TGCGTATAATATTTGCCCATG	−	PCR
CP3	GGATAAAACTTGTGCTTATT	−	Sequencing
JAHL	TGTAGCGTGGCTATATCTCTT	+	PCR

[a] The restriction enzyme site *Xba*I plus four 5′ nucleotides were added to several of the gene cassettes and are shown as lowercase letters in brackets. The 3′ A residue of the *Xba*I sequence in E6CAT, 6JAH4, 6JAH5, 6JAH6, and S6CAT is part of the TGEV genomic sequence. TGEV sequences in the gene cassettes are shown as boldface, uppercase letters, with the potential TASs doubly underlined and nucleotides between the TAS and ATG of the CAT gene singly underlined. Changes to nucleotides within the TASs are shown as lowercase letters. Nucleotides corresponding to the CAT gene are uppercase, italic letters, with the ATG doubly underlined. T7 promoter sequence is in lowercase letters and underlined. All sequences are shown 5′ → 3′.

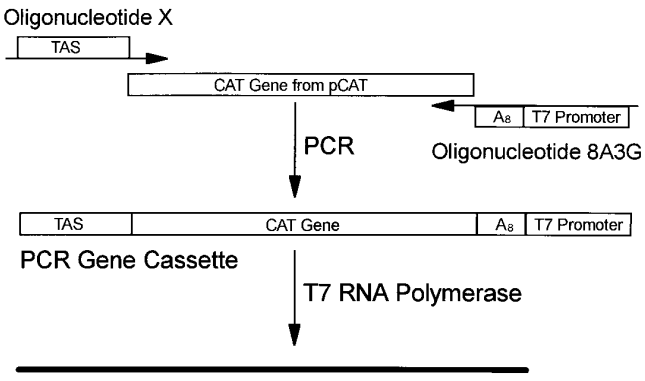

FIG. 1. Schematic representation of the production of CAT reporter gene cassettes from the pCAT basic vector for the synthesis of negative-stranded RNAs to act as potential templates for the TGEV polymerase. Oligonucleotide X corresponds to a series of oligonucleotides (Table 1) containing a TGEV TAS or a modified TAS derived from mRNA 6 or mRNA 7 and the 5′ end of the CAT gene. Oligonucleotide 8A3G corresponds to the 3′ end of the CAT gene, a short poly(A) tail, and the T7 RNA polymerase promoter. The T7 promoter was orientated such that negative copies of the CAT gene with a negative copy of the TGEV TAS sequence at the 3′ end could be produced to act as potential templates for the TGEV polymerase.

itive-sense RNAs, with the TGEV leader attached at the 5′ end, capable of acting as mRNAs for the translation of CAT (Fig. 2). The expression of CAT protein and the presence of a TGEV leader sequence on a CAT mRNA would indicate that the TGEV polymerase had recognized the T7-generated negative-sense RNA transcripts. The T7-generated transcripts have only antisense copies of the CAT gene and are therefore nonfunctional for the synthesis of CAT protein.

In order to test whether in situ-generated negative-sense RNAs could act as templates for synthesis of an mRNA by the TGEV polymerase, the gene cassettes 6CAT and 7CAT (Table 1) were produced by PCR such that the initiation codon of the CAT gene was preceded by the same nucleotides found upstream of the initiation codons of either the TGEV (FS772/70) nucleoprotein (N) gene on mRNA 6 or the ORF-7 gene on mRNA 7 (3). TGEV mRNA 6 and 7 TASs were chosen because previous studies had indicated that mRNA 6 was more abundant than mRNA 7 in TGEV-infected cells (9, 13, 27, 40) and therefore offered a way of examining which nucleotides may play a role in the differential control of mRNA synthesis. The 5′ ends of the PCR-generated gene cassettes were sequenced and found to be as expected from the sequences of the oligonucleotides used in the PCR amplifications. Following transfection into cells coinfected with both TGEV and vTF7-3, the amounts of CAT protein produced from each construct were determined by CAT ELISA. The mean values and standard deviations, from three experiments, for the amounts of

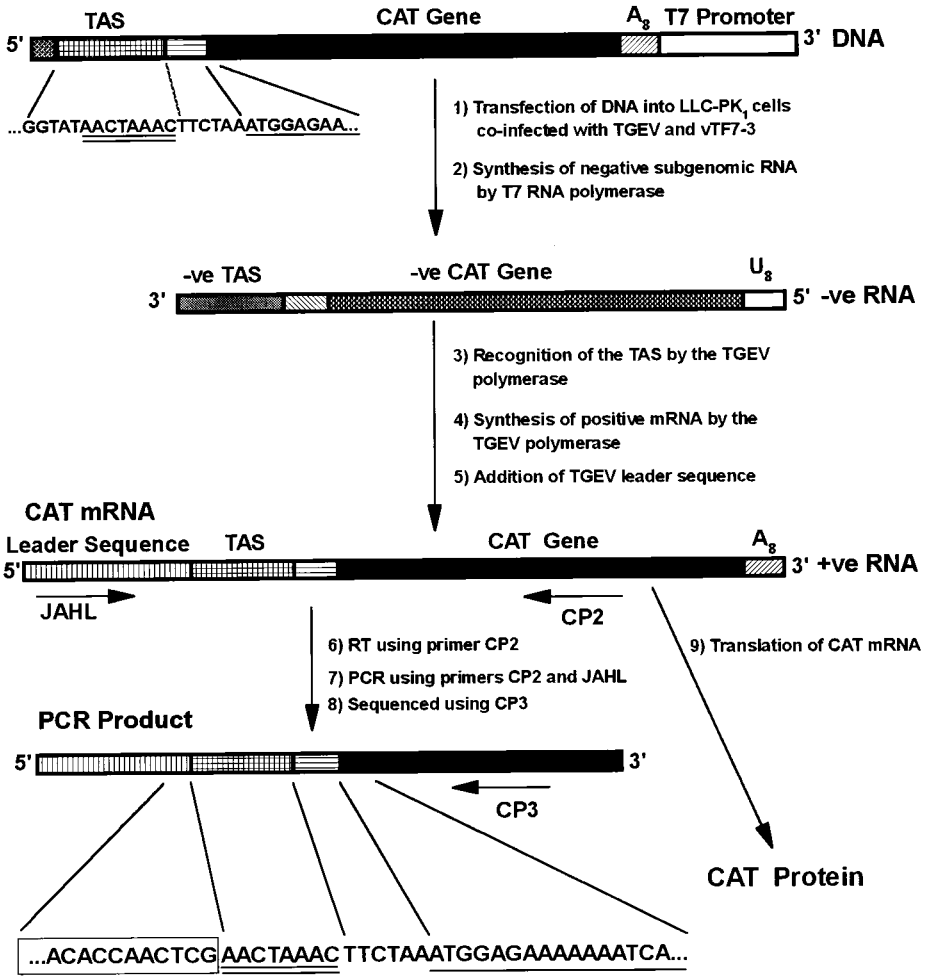

FIG. 2. Schematic representation of the CAT expression protocol. CAT protein can be produced only if the TGEV polymerase recognizes a particular TAS on a negative-sense RNA to initiate transcription of a CAT mRNA. For synthesis of a CAT mRNA via a TGEV TAS (6CAT in this example), it would be predicted that the TGEV leader RNA would be incorporated onto the 5′ end. The sequence at the top represents the TAS, including the flanking nucleotides; the bottom sequence represents part of the theoretical CAT mRNA PCR product in which the TGEV leader RNA has been incorporated at the 5′ end. The singly underlined nucleotides of the sequences represent the 5′ end of the CAT gene, the doubly underlined sequences correspond to the TAS, and the boxed nucleotides on the lower sequence are the 3′ end of the leader sequence.

CAT protein synthesized were 39 ± 5 pg and 25 ± 3 pg for 6CAT and 7CAT, respectively, indicating that 6CAT produced significantly more CAT than 7CAT with a confidence interval of 95%. Constructs S6CAT and S7CAT, which are similar to 6CAT and 7CAT except that the TASs were scrambled (Table 1), did not generate any detectable CAT protein, indicating that the TASs in 6CAT and 7CAT had been utilized for the transcription of CAT mRNA. Constructs that had been transfected into cells that had been infected with only one virus, either TGEV or vTF7-3, or with neither virus did not produce any detectable CAT protein, indicating that both TGEV and vTF7-3 were necessary for the expression of CAT protein.

Analysis of the CAT mRNAs. Total cellular RNA was isolated from cells which had been coinfected with TGEV and vTF7-3 and transfected with the gene cassettes 6CAT, S6CAT, 7CAT, and S7CAT. The RNA was analyzed for the presence of CAT mRNAs containing the TGEV leader RNA sequence by RT-PCR (Fig. 2) with oligonucleotide CP2, which is complementary to an internal sequence (536 nt from the ATG) in the CAT gene (Genbank/EMBL accession no. X65322), for cDNA synthesis. The cDNA was used for PCR amplification with oligonucleotides CP2 and JAHL, which corresponded to a region 20 nt from the 5′ end of the TGEV FS772/70 leader RNA sequence (27). RT-PCR products of the expected size, ≈0.6 kb, were obtained from RNA isolated from cells infected with both TGEV and vTF7-3 and transfected with 6CAT or 7CAT and which had been shown to express CAT protein (data not shown). RT-PCR products were not obtained from cells transfected with S6CAT or S7CAT or from cells transfected with 6CAT or 7CAT but infected only with either TGEV or vTF7-3. Correspondingly, these cells did not contain CAT protein. The 0.6-kb PCR products were sequenced by using oligonucleotide CP3, which is complementary to a region within the CAT gene, 170 nt from the ATG (Fig. 2). Analysis of the sequences revealed that the 5′ ends of the CAT genes were preceded by either the 6CAT or 7CAT TAS and nucleotides corresponding to the TGEV leader sequence (Fig. 3), confirming that the CAT mRNAs had the TGEV leader fused to their 5′ ends (Fig. 4).

The presence of the TGEV leader sequence on the CAT mRNAs following analysis of the RNAs from the transfected cells could have been an artifact from the cDNA and/or PCR

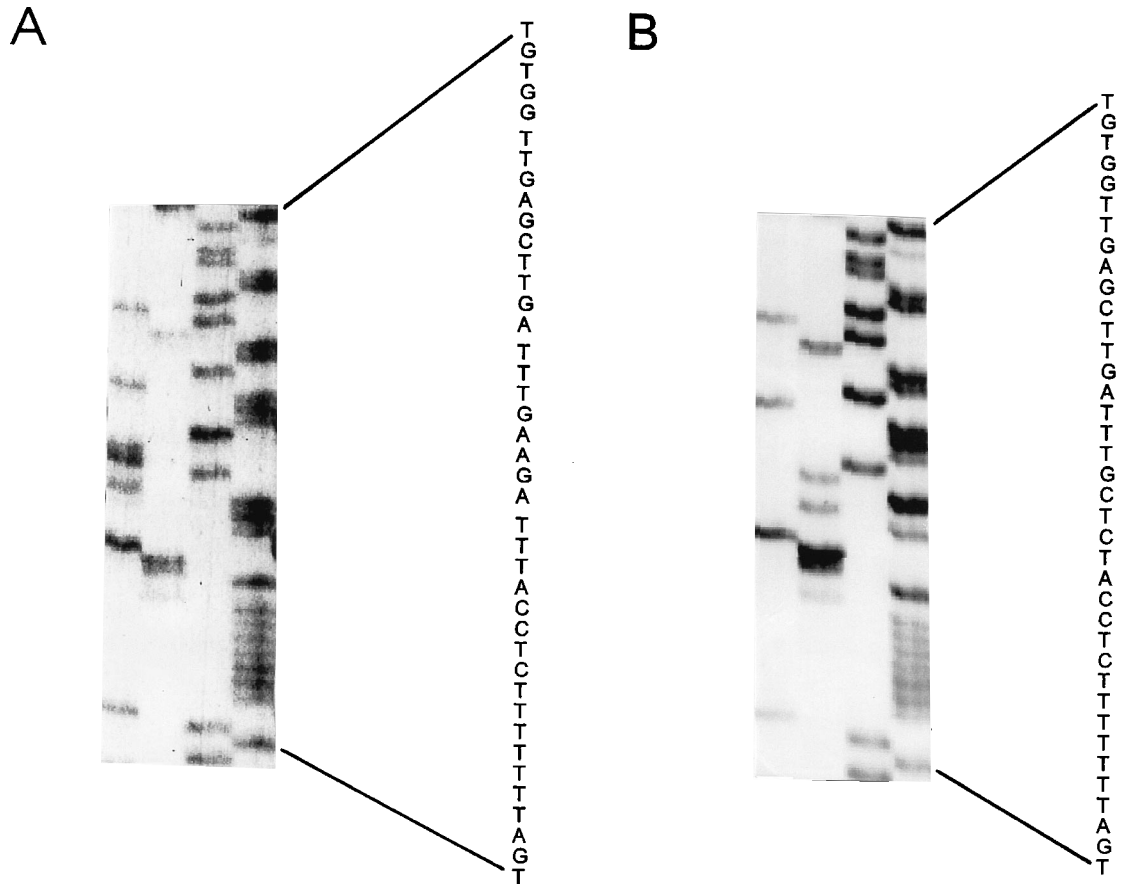

FIG. 3. Sequence analysis of PCR-generated DNA fragments derived from the CAT mRNAs isolated from cells transfected with 6CAT (A) and 7CAT (B). The sequences from bottom to top represent the reverse complements due to the primer used for the sequencing reaction. Therefore, from top to bottom (5′→3′), the sequences are the complements of the mRNA sequences. The uppermost 11 nt (A) and 9 nt (B) represent nucleotides from the TGEV leader sequence, the next 14 nt (A) and 13 nt (B) represent the TAS and 3′ nucleotides for 6CAT and 7CAT, respectively, and the following 16 nt are derived from the CAT gene.

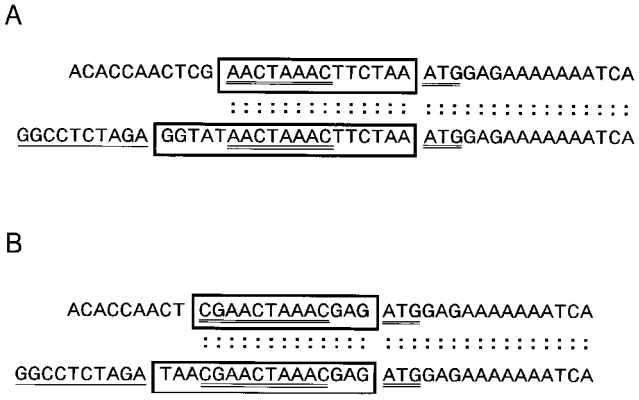

FIG. 4. Alignments of the sequences derived from the CAT mRNAs with the 5′ ends of the PCR fragments, representing constructs 6CAT (A) and 7CAT (B), used to transfect the cells. The top sequences represent the CAT mRNAs, and the bottom sequences represent the 5′ ends of the PCR fragments. The singly underlined sequences represent extra nucleotides, including an *Xba*I site, added for cloning purposes. The boxed sequences represent TGEV genomic nucleotides, including the TAS, which is shown doubly underlined. The initiation codons of the CAT gene are doubly underlined, and the sequences at the 5′ ends of the top sequences, upstream of the TASs, represent the 3′ end of the TGEV leader sequence. Colons are used to show the positions where the nucleotides on the two aligned sequences are the same.

step, e.g., by template switching, due to either the reverse transcriptase or *Taq* polymerase, between the TASs on the T7-derived transcripts and TGEV RNAs. To exclude this possibility RNA was taken from cells which had been infected with vTF7-3 and transfected with either 6CAT or 7CAT and was mixed with an equal quantity of RNA extracted from cells that had been infected with only TGEV. A sample of the mixed RNA was analyzed by RT-PCR under the same conditions used to detect the leader-containing mRNA. No PCR products were detected following analysis by agarose gel electrophoresis and ethidium bromide staining.

In vitro translation of CAT from mRNA 6 and mRNA 7 TASs. The reporter gene system relied upon the expression of CAT protein as an indicator of transcription. To determine whether the larger amount of CAT protein derived from 6CAT compared with that derived from 7CAT was a result of differences in the amounts of mRNA synthesized or of differences in the efficiencies of translation of CAT protein, two new reporter gene constructs were produced. Oligonucleotides m6T7 and m7T7 (Table 1) were synthesized for the production of reporter gene cassettes by PCR amplification from the pCAT-Basic Vector in conjunction with oligonucleotide CAT2. The resulting constructs were used for the in vitro synthesis of positive-sense RNA with the TGEV sequences AACUAAAC UUCUAAAUG and AACUAAACGAGAUG, which were derived from mRNAs 6 and 7, respectively, upstream of the

second codon of the CAT gene and equivalent to the TASs present on the CAT mRNAs derived from the T7-generated negative-sense RNA transcripts. Oligonucleotide CAT2 contained 25 T residues, to produce a poly(A) tail in the resultant RNAs, a length reported to be sufficient for function in both animal and plant cells (8). The AUG of the CAT gene in m6T7 was within a suboptimal context, UAAAUGG, for translation according to Kozak, with the pyrimidine at the −3 position resulting in a context sequence less favorable than the optimal Kozak sequence (A/G)CCAUGG (17–19). In contrast, the Kozak sequence around the AUG of the CAT gene in m7T7, GAGAUGG, was in a more favorable context. RNA was generated in vitro by using T7 RNA polymerase and translated in rabbit reticulocyte lysates. Over a 10-fold range of RNA content (5 to 50 ng), more CAT protein was expressed from the RNA generated from construct m7T7 (51 to 218 pg) than from that generated by construct m6T7 (37 to 163 pg), as predicted from the Kozak sequences. This was in contrast to the observation for the amounts of CAT protein produced in cells following transfection with 6CAT or 7CAT. Since 6CAT produced more CAT protein in cells despite 6CAT mRNA being less efficiently translated than 7CAT mRNA, this indicated that the amounts of CAT protein produced in cells were due to differences in transcription rather than to differences in translation.

Effects of nucleotide changes upstream of the TASs. The results for the expression of CAT mRNAs from the reporter gene cassettes 6CAT and 7CAT indicated that the system could be used for analyzing the roles of various nucleotides in the transcription of TGEV subgenomic mRNAs. The TGEV-derived sequences in 6CAT and 7CAT varied in three regions such that (i) the degree of complementarity between the TASs and the 3′ end of the TGEV leader sequence, (ii) the type and number of nucleotides between the 3′ end of the TAS and the translation initiation codon, and (iii) the nucleotides upstream of the TASs were different. Therefore, these differences (or a combination of them) between the two TASs might be involved in the differential syntheses of TGEV mRNAs 6 and 7 and offered a way of investigating the synthesis of TGEV mRNAs by using the negative-sense RNA system described above.

Potential base pairing outside a TAS has been postulated to be involved in the binding of the leader RNA to TASs for both MHV (15) and bovine coronavirus (10). The number of nucleotides upstream of the TASs for both 6CAT and 7CAT was increased to 11, to produce extended constructs E6CAT and E7CAT, in order to investigate whether these upstream genome-derived nucleotides would alter the transcription of the CAT reporter gene. Increasing the number of TGEV genomic nucleotides upstream of the mRNA 6 TAS, as in E6CAT, increased the expression of CAT protein approximately twofold compared with that for 6CAT (Fig. 5A), whereas extending the number of genomic nucleotides upstream of the mRNA 7 TAS, as in E7CAT, did not increase the expression of CAT protein compared with that for 7CAT (Fig. 5C). Thus, increasing the number of nucleotides upstream of the TASs to 11 increased the amount of CAT protein synthesized for 6CAT but not for 7CAT.

To investigate the potential role of the other regions, several constructs in which modified versions of the TGEV mRNA 6 or mRNA 7 TASs were placed upstream of the CAT gene were generated (Table 1). Essentially, various nucleotides within or flanking the mRNA 6 and 7 TASs were interchanged. Constructs were synthesized by PCR and transfected into cells previously coinfected with TGEV and vTF7-3, and the cell extracts were analyzed for the amounts of CAT protein. The resulting data were analyzed by the statistical analysis package Generalized Linear Interactive Modelling (GLIM), version 3.72 (1), to determine whether there were any significant variations in the amounts of CAT protein produced from the modified mRNA 6 constructs and the modified mRNA 7 constructs compared with those produced from E6CAT and 7CAT, respectively. GLIM analysis indicated that there were no significant variations in the mean values obtained from four experiments at the 5% level, and therefore the data were pooled for a more comprehensive analysis. The data generated by GLIM are represented as a bar chart (Fig. 5) in which 95% confidence intervals are indicated by the vertical lines and mean values are significantly different when the confidence interval lines do not overlap.

Effects of nucleotide changes within the TASs. Increasing the potential base pairing of the mRNA 6 TAS with the 3′ end of the leader sequence from 8 to 10 nt (6JAH5), as found for the mRNA 7 TAS, did not significantly affect the amount of CAT produced compared with that for E6CAT (Fig. 5A). Similarly, decreasing the number of nucleotides in the mRNA 7 TAS from 10 to 8 (7JAH1) did not significantly affect the amount of CAT protein produced compared with that for 7CAT (Fig. 5B). However, reducing the mRNA 6 TAS from 8 to 6 nt (AACTAAAC to CTAAAC; 6JAH6), to give the minimal predicted TGEV TAS as found for mRNA 4, significantly decreased (by approximately threefold) the amount of CAT produced compared with that for E6CAT (Fig. 5A).

Effects of nucleotide changes between the TASs and AUG. The results showed that changing the nucleotides between the mRNA 6 TAS and the AUG of the CAT gene from UUCUAA to GAG (6JAH4), as found with mRNA 7, significantly decreased (approximately 2.5-fold) the amount of CAT produced compared with that for E6CAT (Fig. 5A). Conversely, changing the nucleotides between the mRNA 7 TAS and the CAT AUG codon from GAG to UUCUAA (7JAH3), as found with mRNA 6, significantly increased (approximately 4.7-fold) the amount of CAT produced compared with that for 7CAT (Fig. 5B).

Effect of decreasing the number of nucleotides upstream of the TASs. Increasing the number of nucleotides found upstream of the mRNA 6 TAS as found on the genomic sequence, from 5 to 11 nt (E6CAT), significantly increased the amount of CAT produced (Fig. 5A) compared with that for 6CAT, indicating that these nucleotides may play a regulatory function. To investigate the effect of these genomic nucleotides on transcription, the number of nucleotides found upstream of the TASs in the mRNA 6-derived and modified constructs was reduced to three for each construct (prefix Sh in Table 1). Cells were coinfected with TGEV and vTF7-3 and transfected with either 6CAT, E6CAT, Sh6CAT, Sh6JAH4, Sh6JAH5, or Sh6JAH6. To minimize any experimental variations, the experiments were repeated five times. The shortened constructs resulted in the same amounts of CAT protein as their longer counterparts, except for E6CAT compared with either 6CAT or Sh6CAT (Fig. 5C).

Analysis of CAT mRNAs. The detection of CAT protein in infected cells that had been transfected with the TAS-containing reporter gene cassettes indicated that CAT mRNAs had been produced from the T7-generated negative-sense RNA transcripts. Both the leader-primed transcription and the termination-extension hypotheses (32) predict that these mRNAs should contain a TGEV-derived leader sequence if they were produced in a manner analogous to that for coronavirus subgenomic mRNAs. However, because of the nature of the negative-sense system used, the production of an mRNA with a TGEV leader sequence fused to the 5′ end could result from either of the two main transcription models. To determine

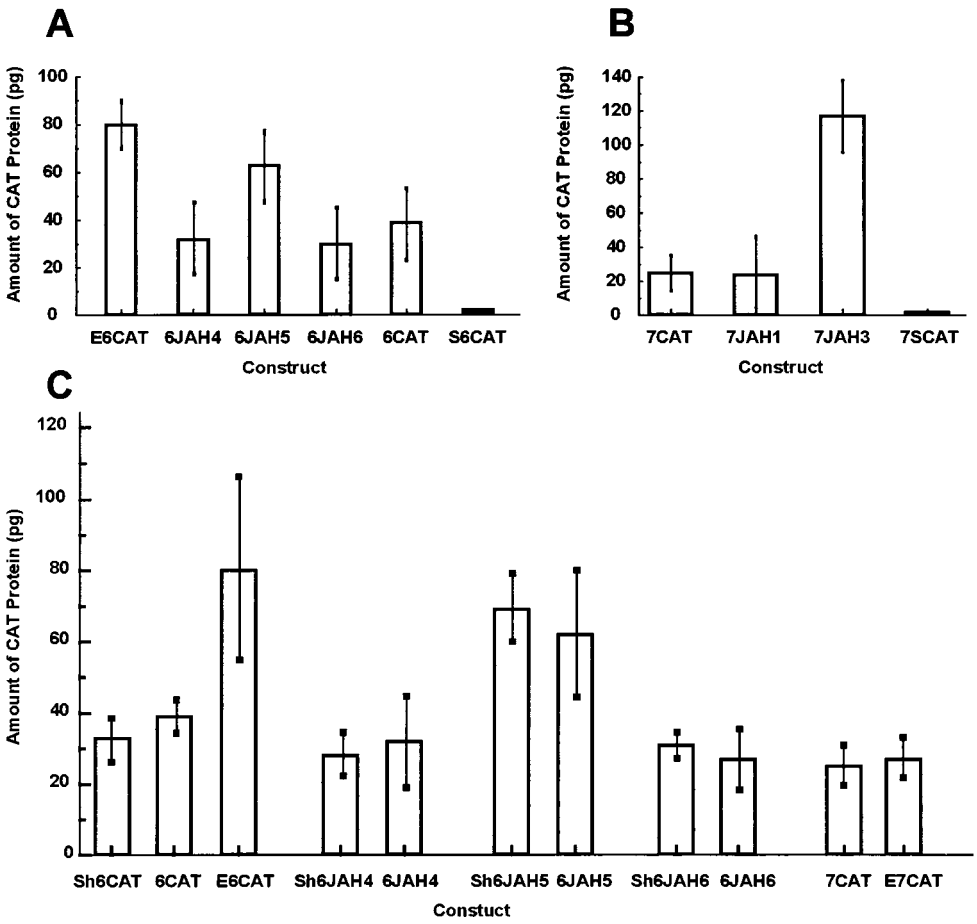

FIG. 5. Analysis of the amounts of CAT protein generated by the various reporter gene cassettes by using GLIM for modified mRNA 6 constructs compared with E6CAT (A) and modified mRNA 7 constructs compared with 7CAT (B). The 95% confidence intervals (not standard deviations) are indicated by the vertical lines; the mean values are significantly different when the confidence interval lines do not overlap. Comparisons can be made only within the mRNA 6 TAS group or within the mRNA 7 TAS group and not between the two groups. The data were from four experiments and a total of 10 samples. (C) Means and standard deviations (σ_{n-1}) of the amounts of CAT protein produced from the shortened (prefix Sh), normal (no prefix), and extended (prefix E) gene constructs as determined from five experiments.

whether helper virus-derived leader sequence participated in the production of CAT mRNAs, cytoplasmic RNA was extracted from cells that had been coinfected with TGEV and vTF7-3 and transfected with either 6CAT, 7CAT, 6JAH4, 6JAH5, 6JAH6, 7JAH1, or 7JAH3. cDNA was synthesized by using oligonucleotide CP2 and used for PCR amplifications with oligonucleotides CP2 and JAHL. Sequence analysis of the products, by either direct PCR sequencing or sequencing of TA-cloned PCR products, revealed that the CAT mRNAs contained sequences corresponding to the TGEV FS772/70 leader sequence (27). All of the CAT mRNAs sequenced had the sequence .....CACCAACU[TAS][X]AUGGAGAAAAAA AUCA at their 5′ ends, in which .....CACCAACU corresponded to the 3′ end of the leader sequence, **[TAS]** corresponded to either AACUAAAC or CGAACUAAAC in the TASs of mRNAs 6 or 7, respectively, **[X]** corresponded to the nucleotides between the TASs and AUG of the CAT gene (UUCUAA for mRNA 6 and GAG for mRNA 7), and the rest of the nucleotides corresponded to the 5′ end of the CAT gene. The results showed that the nucleotides 5′ to the TASs in the PCR gene cassettes, corresponding to both TGEV genome-derived nucleotides and those both constituting and upstream of the *Xba*I restriction site (Table 1), had been replaced by the TGEV leader sequence.

DISCUSSION

We have developed a model system to study the differential control of transcription of coronavirus mRNAs. This study is the first to utilize a negative-sense RNA for the expression of a reporter gene under the control of a coronavirus transcription-replication system and allowed us to test for the production of mRNAs by the TGEV polymerase, in two aspects: (i) the expression of the reporter gene product (in this case, CAT) and (ii) whether the new mRNA was coronavirus-like, i.e., had a leader RNA sequence at the 5′ end, which is the case for all coronavirus mRNAs. We have demonstrated that CAT protein was expressed, in TGEV-infected cells, from a negative-sense RNA provided that the RNA contained a negative-sense TGEV TAS, allowing us to use the system for studying the roles of various nucleotides, within and around the TASs, in the transcription of TGEV mRNAs.

Earlier work with MHV had indicated that the extent of complementarity between the leader and the TASs of the subgenomic mRNAs might regulate the expression of different mRNA species (4, 36). Our results indicated that differences in the potential degree of base pairing between the TASs, as found for mRNAs 6 and 7, and the 3′ end of the leader RNA sequence had no significant effect on transcription. However,

reduction of the potential base pairing of the TGEV TAS from 7 nt, as found for most TGEV mRNAs, to 6 nt, as found for the low-abundance TGEV mRNA 4, did significantly decrease the amount of CAT protein expressed. This observation together with the observation that scrambled TASs did not lead to the expression of CAT would indicate that some potential base pairing between the TASs and the leader sequence is essential for transcription and/or that specific nucleotide sequence-protein interactions are required. Our observations indicated that increasing the potential base pairing between the TASs and leader sequence beyond that achieved with the sequence ACU AAAC did not enhance transcription. These data argue against the hypothesis that the degree of complementarity between the TAS and the leader sequence regulates transcription of a subgenomic mRNA. Similar findings from a study in which an MHV defective-interfering RNA, containing TASs corresponding to MHV mRNAs 3 and 7, was used to study transcription have recently been described (39). Although the MHV mRNA 3- and 7-derived TASs have base pairings of 10 and 17 nt, respectively, with the MHV leader RNA, the amounts of subgenomic mRNAs generated from the defective-interfering RNA were similar. Those authors concluded that base pairing, between the TASs and the leader RNA, did not control mRNA abundance, although some residual base pairing was essential. They also suggested that transcription initiation might require recognition of the TAS by the transcriptase.

Initial experiments in which the number of genome-derived nucleotides upstream of the TAS sequence in 6CAT was increased from 5 to 11 (E6CAT) significantly increased the production of CAT. This indicated that sequences upstream of the TAS, which are not expected to interact with the leader RNA, may influence the control of transcription. However, increasing the number of nucleotides 5′ of the TAS in the mRNA 7-based construct, 7CAT, did not alter the level of transcription, nor did decreasing the number of nucleotides from 5 to 3 (Sh6CAT) upstream of the mRNA 6 TAS (Fig. 5C). These observations together with the results obtained for increasing the number of nucleotides upstream of the mRNA 7 TAS indicate that nucleotides, or at least the first 11 nt, upstream of the TASs do not play a role in the control of transcription. This observation supports the results obtained by Makino and Joo (25), who concluded that non-base-paired flanking sequences did not play a role in the transcription of MHV subgenomic mRNAs. The increase in transcription observed for E6CAT compared with 6CAT was probably a phenomenon associated with the E6CAT construct and was possibly due to alterations in secondary structures. It should be noted that we cannot discount the possibility that nucleotides further upstream of the TASs on the genomic RNA may influence transcription levels and that these also have yet to be investigated in the defective-interfering systems.

Budzilowicz et al. (4) suggested that nucleotides between the TAS and the AUG of the coding sequence may play a role in the control of transcription. Hofmann et al. (10) proposed for bovine coronavirus that base pairing outside the region of the leader-mRNA junction may contribute to transcription initiation. We found that the sequence between the TAS and the AUG of the CAT gene did influence the level of transcription. Whether this was by the alteration of the potential secondary structure of the RNA or by the alteration of some, as-yet-unknown, control element is not known. An alternative explanation was that the alterations might have increased the efficiency of translation rather than increasing transcription because of potential alterations to the Kozak sequence around the CAT AUG. The Kozak sequence was changed from UAAAUGG (E6CAT) to GAGAUGG in 6JAH4 and from GAGAUGG (7CAT) to UAAAUGG in 7JAH3. The change from E6CAT to 6JAH4 represented a change to a more favorable Kozak sequence, but the amount of CAT detected from 6JAH4 was less than that from E6CAT. The change from 7CAT to 7JAH3 represented a change to a less favorable Kozak sequence, but the amount of CAT detected from 7JAH3 was more than that from 7CAT. These results were the opposite of those that would have been predicted if the levels of translation observed depended on the Kozak sequences and were the opposite of those observed from the in vitro expression of RNA derived from constructs m6T7 and m7T7, favoring a change in the level of transcription as an explanation. It is possible that by increasing the efficiency of translation from mRNA 7, this may compensate for the low level of transcription of mRNA 7 observed in TGEV-infected cells.

Zhang et al. (41) have suggested that a number of different components are involved in the regulation of MHV subgenomic mRNA transcription: the TAS, a *trans*-acting leader RNA, a spacer sequence (5′ untranslated region and MHV gene 1 sequence), and a *cis*-acting leader RNA. Our results confirm that a *trans*-acting leader RNA and a TAS are required for the transcription of CAT mRNAs from the reporter genes. However, a *cis*-acting sequence and spacer sequence were not prerequisites for transcription of the CAT reporter genes in the system described in this paper. Our constructs contained only TGEV nucleotides derived from genomic sequence corresponding to up to 11 nt upstream of the TAS, the TAS itself, and nucleotides between the TAS and the second codon of the CAT gene. However, as Liao and Lai (24) suggested, the *cis*-acting element may act as an enhancer of transcription, and the addition of a TGEV *cis*-acting element to our reporter gene constructs might result in an up regulation of transcription.

Sequence data confirmed that all of the CAT mRNAs sequenced contained the TGEV leader sequence, and no heterogeneity of the leader-mRNA junction was observed. Because the reporter gene cassettes contained only nucleotides within the TASs corresponding to the leader RNA sequence, the TGEV leader sequence must have been derived from the virus, i.e., added in *trans*. Constructs 6JAH6 and 7JAH1 contained nucleotide substitutions corresponding to and therefore not derived from genomic sequence at the 5′ end of the TASs, reducing the potential degree of complementarity between the TAS and the 3′ end of the leader sequence by two bases, i.e., from AACUAAAC to UUCUAAAC for 6JAH6 and from CGAACUAAAC to UCAACUAAAC for 7JAH1. Sequence analysis of the corresponding CAT mRNAs showed that the nucleotide substitutions had been replaced by the authentic TAS nucleotides, indicating that they had been derived from the helper virus leader sequence and that the leader RNA was fused downstream of the substitutions. This observation supports the prediction of van der Most et al. (39) that transcription initiation upstream of a mutation in a TAS would result in mRNAs carrying that mutation, whereas initiation downstream would give rise to transcripts containing the authentic leader sequence. Our observation that the nucleotides 5′ to the TASs were replaced on the mRNAs would indicate that the leader sequence fused to the RNA within the TAS, presumably at the ACUAAAC sequence, according to the leader-priming hypothesis. Alternatively, if the anti-TAS on the negative-sense RNA template recognized the TAS of the leader RNA on the genomic RNA, according to the differential termination hypothesis, the 3′ non-base-pairing nucleotides must have been removed, as postulated for trimming of the free leader RNA in the leader-priming hypothesis. The resulting nascent negative-

sense RNA, and subsequent positive-sense mRNA copies, would therefore not contain the incorrect nucleotides.

The system that we used to study the synthesis of a CAT mRNA could have operated by either of the discontinuous mechanisms described in the introduction. Previous studies had shown the expression of reporter genes (41) or the synthesis of subgenomic mRNAs (25, 26, 39) from TAS sequences inserted into modified defective replicating RNAs. In these systems the RNA contained sequences derived from both the 5′ and 3′ ends of the genome which are believed to be required for replication of coronavirus RNA. No such sequences were present on the template RNA in our system, indicating that synthesis of the CAT mRNA, and potentially that of coronavirus subgenomic mRNAs, can be initiated by using certain common nucleotides found upstream of each coronavirus gene. It is probable that both mechanisms are involved in the transcription of coronavirus mRNAs. The potential identification of a negative-sense RNA with an antileader in our system would indicate that negative-sense leader-containing templates play a role in the production of coronavirus subgenomic mRNAs but would not preclude a role of leader priming. We are currently investigating whether negative-sense RNAs containing antileader sequences are present in our system.

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REFERENCES

1. **Baker, R. J., M. R. B. Clarke, and J. A. Nelder.** 1978. Generalized linear interactive modelling (GLIM), version 3.72, update 0. The Royal Statistical Society, London.
2. **Brian, D. A., R.-Y. Chang, M. A. Hofmann, and P. B. Sethna.** 1994. Role of subgenomic minus-strand RNA in coronavirus replication. Arch. Virol. **9**(Suppl.)**:**173–180.
3. **Britton, P., R. S. Carmenes, K. W. Page, D. J. Garwes, and F. Parra.** 1988. Sequence of the nucleoprotein from a virulent British field isolate of transmissible gastroenteritis virus and its expression in *Saccharomyces cerevisiae*. Mol. Microbiol. **2:**89–99.
4. **Budzilowicz, C. J., S. P. Wilcynski, and S. R. Weiss.** 1985. Three intergenic regions of coronavirus mouse hepatitis virus strain A59 genome RNA contain a common nucleotide sequence that is homologous to the 3′ end of the viral mRNA leader sequence. J. Virol. **53:**834–840.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **De Groot, R. J., R. J. ter Haar, M. C. Horzinek, and B. A. van der Zeijst.** 1987. Intracellular RNAs of the feline infectious peritonitis coronavirus strain 79-1146. J. Gen. Virol. **68:**995–1002.
7. **Fuerst, T. R., P. L. Earl, and B. Moss.** 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. **7:**2538–2544.
8. **Gallie, D. R., J. N. Feder, R. T. Schimike, and V. Walbot.** 1991. Posttranslational regulation in higher eukaryotes: the role of the reporter gene in controlling expression. Mol. Gen. Genet. **228:**258–264.
9. **Hiscox, J. A., D. Cavanagh, and P. Britton.** 1995. Quantification of individual subgenomic mRNA species during replication of transmissible gastroenteritis virus. Virus Res. **36:**119–130.
10. **Hofmann, M. A., R.-Y. Chang, S. Ku, and D. A. Brian.** 1993. Leader-mRNA junction sequences are unique for each subgenomic mRNA species in the bovine coronavirus and remain so throughout persistent infection. Virology **196:**163–171.
11. **Horsburgh, B. C., I. Brierley, and T. D. K. Brown.** 1992. Analysis of a 9.6kb sequence from the 3′ end of canine coronavirus genomic RNA. J. Gen. Virol. **73:**2849–2862.
12. **Hu, G.** 1993. DNA polymerase-catalysed addition of nontemplated extra nucleotides to the 3′ end of a DNA fragment. DNA Cell Biol. **12:**763–770.
13. **Jacobs, L., B. A. M. Van Der Zeijst, and M. C. Horzinek.** 1986. Characterization and translation of transmissible gastroenteritis virus mRNAs. J. Virol. **57:**1010–1015.
14. **Jeong, Y. S., and S. Makino.** 1992. Mechanism of coronavirus transcription: duration of primary transcription initiation activity and effects of subgenomic RNA transcription on RNA replication. J. Virol. **66:**3339–3346.
15. **Joo, M., and S. Makino.** 1992. Mutagenic analysis of the coronavirus intergenic consensus sequence. J. Virol. **66:**6330–6337.
16. **Konings, D. A. M., P. J. Bredenbeek, J. F. H. Noten, P. Hogeweg, and W. J. M. Spaan.** 1988. Differential premature termination of transcription as a proposed mechanism for the regulation of coronavirus gene expression. Nucleic Acids Res. **16:**10849–10860.
17. **Kozak, M.** 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol. Rev. **47:**1–45.
18. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44:**283–292.
19. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108:**229–241.
20. **Lai, M. M. C.** 1986. Coronavirus leader-RNA-primed transcription: an alternative mechanism to RNA splicing. Bioessays **5:**257–260.
21. **Lai, M. M. C.** 1990. Coronavirus—organization, replication and expression of genome. Annu. Rev. Microbiol. **44:**303–333.
22. **Lai, M. M. C., C.-L. Liao, Y.-J. Lin, and X. Zhang.** 1994. Coronavirus: how a large RNA viral genome is replicated and transcribed. Infect. Agents Dis. **3:**98–105.
23. **Lai, M. M. C., C. D. Patton, R. S. Baric, and S. A. Stohlman.** 1983. Presence of leader sequences in the mRNA of mouse hepatitis virus. J. Virol. **46:**1027–1033.
24. **Liao, C.-L., and M. M. C. Lai.** 1994. Requirement of the 5′-end genomic sequence as an upstream *cis*-acting element for coronavirus subgenomic mRNA transcription. J. Virol. **68:**4727–4737.
25. **Makino, S., and M. Joo.** 1993. Effect of intergenic consensus sequence flanking sequences on coronavirus transcription. J. Virol. **67:**3304–3311.
26. **Makino, S., M. Joo, and J. K. Makino.** 1991. A system for study of coronavirus mRNA synthesis: a regulated, expressed subgenomic defective interfering RNA results from intergenic site insertion. J. Virol. **65:**6031–6041.
27. **Page, K. W., P. Britton, and M. E. G. Boursnell.** 1990. Sequence analysis of the leader RNA of two porcine coronaviruses: transmissible gastroenteritis virus and porcine respiratory coronavirus. Virus Genes **4:**289–301.
28. **Page, K. W., K. L. Mawditt, and P. Britton.** 1991. Sequence comparison of the 5′-end of mRNA 3 from transmissible gastroenteritis virus and porcine respiratory coronavirus. J. Gen. Virol. **72:**579–587.
29. **Pulford, D. J., and P. Britton.** 1991. Intracellular processing of the porcine coronavirus transmissible gastroenteritis virus spike protein expressed by recombinant vaccinia virus. Virology **182:**765–773.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Sawicki, S. G., and D. L. Sawicki.** 1986. Coronavirus minus-strand RNA synthesis and effect of cycloheximide on coronavirus RNA synthesis. J. Virol. **57:**328–334.
32. **Sawicki, S. G., and D. L. Sawicki.** 1990. Coronavirus transcription: subgenomic mouse hepatitis virus replicative intermediates function in RNA synthesis. J. Virol. **64:**1050–1056.

32a. **Schaad, M. C., and R. S. Baric.** 1994. Genetics of mouse hepatitis virus transcription: evidence that subgenomic negative strands are functional templates. J. Virol. **68:**8169–8179.

33. **Schenborn, E. T., and R. C. Mierendorf.** 1985. A novel transcription property of SP6 and T7 RNA polymerases: dependence on template structure. Nucleic Acids Res. **13:**6223–6236.
34. **Sethna, P. B., M. A. Hofmann, and D. A. Brian.** 1991. Minus-strand copies of replicating coronavirus mRNAs contain antileaders. J. Virol. **65:**320–325.
35. **Sethna, P. B., S. L. Hung, and D. A. Brian.** 1989. Coronavirus subgenomic minus-strand RNAs and the potential for mRNA replicons. Proc. Natl. Acad. Sci. USA **86:**5626–5630.
36. **Shieh, C. K., L. H. Soe, S. J. Makino, M. F. Chang, S. A. Stohlman, and M. M. C. Lai.** 1987. The 5′-end sequence of the murine coronavirus genome—implications for multiple fusion sites in leader-primed transcription. Virology **156:**321–330.
37. **Spaan, W., D. Cavanagh, and M. C. Horzinek.** 1988. Coronaviruses: structure and genome expression. J. Gen. Virol. **69:**2939–2952.
38. **Spaan, W. J. H., H. Delius, M. Skinner, J. Armstrong, P. Rottier, S. Smeekens, B. A. M. Van der Zeijst, and S. G. Siddell.** 1983. Coronavirus mRNA synthesis involves fusion of non-contiguous sequences. EMBO J. **2:**1839–1844.
39. **van der Most, R. G., R. J. de Groot, and W. J. M. Spaan.** 1994. Subgenomic RNA synthesis directed by a synthetic defective interfering RNA of mouse hepatitis virus: a study of coronavirus transcription initiation. J. Virol. **68:**3656–3666.
40. **Wesley, R. D., A. K. Cheung, D. D. Michael, and R. D. Woods.** 1989. Nucleotide sequence of coronavirus TGEV genomic RNA: evidence for 3 mRNA species between the peplomer and matrix protein genes. Virus Res. **13:**87–100.
41. **Zhang, X., C.-L. Liao, and M. M. C. Lai.** 1994. Coronavirus leader RNA regulates and initiates subgenomic mRNA transcription both in *trans* and in *cis*. J. Virol. **68:**4738–4746.