

Quantification of individual subgenomic mRNA species during replication of the coronavirus transmissible gastroenteritis virus

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Abstract

A biotinylated-oligonucleotide-based method was used to isolate the subgenomic mRNAs of the coronavirus transmissible gastroenteritis virus (TGEV) to investigate the amounts of the mRNAs produced at early, middle and late times in the replication cycle. TGEV mRNA 6, which encodes the N protein, was observed to be the most abundant species throughout the replication cycle. The ratios of mRNA 6 to the other mRNAs were 1:0.11 (mRNA 2), 1:0.16 (mRNAs 3 and 4) and 1:0.37 (mRNA 5) at 12 h post-infection. All the mRNA species were differentially regulated throughout the replication cycle, although the rate of accumulation of mRNAs 4, 5 and 6, but not mRNA 3, increased markedly towards the end of the replication cycle. mRNA 7 was not detected in the system used. There was no observable correlation between the amounts of each mRNA synthesised and the potential degree of base pairing between the 3' end of the leader sequence and the transcription associated sequences on the genomic RNA at any time during the replication cycle. This indicates that the extent of base pairing was not the only factor involved in the control of subgenomic mRNA synthesis.

Keywords: TGEV; Coronavirus; Porcine; mRNA; Leader RNA; Transcription

1. Introduction

Transmissible gastroenteritis virus (TGEV), a member of the genus *Coronavirus*, family *Coronaviridae*, is an enveloped virus with a single-stranded,

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positive-sense RNA genome, of approximately 28,000 nucleotides (nts), 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 (Jacobs et al., 1986; Wesley et al., 1989; Page et al., 1990). Although most of the mRNAs contain more than one gene only the 5'-most gene(s) not present on the next smallest mRNA is translated. Most coronavirus genes, on the genomic RNA, are preceded by a non-translated region of varying length, the intergenic sequence (Lai, 1990). On the negative-stranded copy of the genomic RNA these intergenic sequences may act as initiation sites for the transcription of the subgenomic mRNAs. In this paper we shall refer to the sequences involved in the synthesis of the mRNAs as the transcription associated sequences (TASSs). In addition to the virus-specific mRNAs, subgenomic negative-stranded RNAs, which correspond to their respective subgenomic mRNA, have been identified in cells infected with TGEV (Sethna et al., 1989), bovine coronavirus (BCV) (Hofmann et al., 1990) and mouse hepatitis virus (MHV) (Sawicki and Sawicki, 1990), and for TGEV have been shown to contain an anti-leader sequence at the 3' end and a short poly(U) sequence at the 5' end (Sethna et al., 1989, 1991). The role of the subgenomic negative-strand RNAs is still not understood, with some authors indicating that they play an important role in coronavirus mRNA synthesis (Sethna et al., 1989; Sawicki and Sawicki, 1990) and others that they play only a small, if any, role and are the end products of the transcription and/or replication process (Jeong and Makino, 1992).

The transcriptional strategy for coronaviruses is complex and at present not completely understood. To date the leader-primed hypothesis has been predominantly used to explain the origin of the subgenomic mRNAs in which a *trans*-acting leader RNA, derived from the 3' end of the negative-strand copy of the genomic RNA, recognises conserved sequences on the negative-strand of the genomic RNA for the transcription of the subgenomic mRNAs (Lai and Stohlman, 1978; Yokomori et al., 1992). The conserved sequences (TASSs) are found proximal to all coronavirus genes though the sequences vary between the different coronaviruses and even between different genes of a coronavirus.

In general coronavirus mRNAs are synthesised in decreasing amounts as the size of the species increases (Lai et al., 1984; Shieh et al., 1987). However, analysis of the mRNA species, usually at a single time point in the replication cycle, by either metabolic labelling or Northern hybridization, produced by the TGEV group of coronaviruses, TGEV (Jacobs et al., 1986; Sethna et al., 1989; Page et al., 1990), porcine respiratory coronavirus (PRCV) (Page et al., 1991), feline infectious peritonitis virus (FIPV) (de Groot et al., 1987) and canine coronavirus (CCV) (Horsburgh et al., 1992), indicated that they were not synthesized in amounts inversely related to their length, implying there may be some control mechanism for the transcription of particular mRNAs.

The leader RNA sequences for TGEV, strains FS772/70 and Purdue, are about 90 nts long (Page et al., 1990; Sethna et al., 1991) and sequence data has indicated that the TGEV leader RNA recognises the minimal sequence, CUA AAC, proximal to each gene (Table 1). Analysis of the subgenomic mRNAs from cells infected

Table 1
Comparison of TGEV (FS772/70) TAS sites

mRNA junction	Intergenic sequence	Base pairing
2 (S)	UAAG <u>UUAACUAAAC</u> UUUGGUAACCACUUCGUUACACACCAUG	7
3	UUAAGA <u>ACUAAAC</u> UUUCAAGUCAUUACAGGUCCUGUAUG	9
4 (sM)	GGCGGUUC <u>UAAACG</u> AAAUUGACUUAAAAAGAAGAAGAGGGAG ACCGUACC <u>UAUG</u>	6
5 (M)	GUUUGAACUAAACAAA <u>UAUG</u>	9
6 (N)	GGUAUAACUAAACUUCUAA <u>UAUG</u>	8
7	UAACGAACUAAACGAGA <u>UAUG</u>	10

Note. Sequences underlined (TASs) show the nucleotides that potentially base pair with the 3' end of the leader RNA. The initiation codons for the respective genes are in boldface. The 3' end of the leader RNA is ACUCGAACUAAAC as determined by Page et al. (1990). The mRNA species encoding the TGEV structural proteins are indicated.

with the Miller strain of TGEV showed the presence of an additional mRNA species, mRNA 3-1. This was postulated to be synthesised from a TAS, CUA AAC, proximal to the potential TGEV ORF-3b gene (Wesley et al., 1989). The mRNA 3 of PRCV, a variant of TGEV, was observed to be smaller than the equivalent TGEV mRNA due to the loss of the original TAS, found upstream of ORF-3a in TGEV, and the creation of a new TAS, ACUAAAC, upstream of the PRCV (strains 86/137004 and 86/135308) ORF-3 gene (Page et al., 1991). The ratios of the PRCV mRNAs appeared to be similar to those observed for TGEV, in which the amount of PRCV mRNA 3 was similar to the amount of TGEV mRNA 3, produced from the TAS proximal to the potential TGEV ORF-3a gene, but higher than the amount observed for the Miller mRNA 3-1. The only difference between the TAS for mRNA 3-1 of the TGEV Miller strain and that of PRCV mRNA 3 was the presence of the extra adenosine nt at the 5' end of the ACUAAAC sequence of PRCV. Interestingly the TAS of mRNA 3-1 has the same sequence, CUA AAC, as for mRNA 4, for all strains of TGEV sequenced to date, and observed to be produced in lower amounts than TGEV mRNA 3. These observations indicated that the base pairing between the TGEV TASs and the leader RNA might play some role in the control of mRNA transcription.

We have investigated whether individual TGEV mRNA species were produced at specific times during the replication cycle and if there was any correlation between the extent of leader-TAS base pairing and the amounts of each mRNA produced at early, middle and late time points.

2. Materials and methods

2.1. Radioactive labelling of TGEV RNA

TGEV, strain FS772/70, was grown in LLC-PK₁ cells in medium containing 1% Eagles MEM (ICN Flow), 0.3% tryptose phosphate broth (Difco), 0.02% yeast

extract, 0.2% sodium bicarbonate, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 25 units/ml mycostatin, 30 mM N-[2-hydroxyethyl]piperazine-N'-2-[ethanesulphonic acid] (HEPES; Sigma) and 10 $\mu\text{g}/\text{ml}$ trypsin (Difco 1:250) (Hofmann and Wyler, 1988). The virus was titrated by plaque assay. Flasks (25 cm^2) of LLC-PK₁ cells were inoculated with TGEV at an m.o.i. of 10 and incubated at 37°C for 1 h. The cells were washed and incubated with fresh medium and at hourly intervals, 5 to 13 h p.i., the medium was removed from some flasks and replaced with medium containing 1 $\mu\text{g}/\text{ml}$ actinomycin D (Calbiochem) and 7.4 MBq/ml 5,6-[³H]uridine (NEN-Dupont NET-367, 1.30–1.85 TBq/mM). After 2 h total cellular RNA was isolated using the method of Chomczynski and Sacchi (1987).

2.2. Synthesis and purification of biotinylated oligonucleotides

Oligonucleotide, biotin-GTATATCACTATCAAAAAGGAAAA (B-153), which was complementary to the 3' end of the TGEV genome (Britton et al., 1988) was synthesized on an Applied Biosystems 381A DNA synthesizer and purified by gel electrophoresis (Sambrook et al., 1989). The biotin residue was incorporated at the 5' end using biotin-phosphoramidite (Cruachem).

2.3. Sequence-specific isolation of TGEV RNA

Streptavidin magnetic beads (Dynabeads® M-280, Dynal®) were prepared by washing with 0.1 M NaOH, 0.5 M NaCl followed by 0.1 M NaCl and binding-washing (BW) buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA and 2 M NaCl). Oligonucleotide B-153 (200 pmols) was coupled to 1 mg of streptavidin magnetic beads in BW buffer for 15 min at room temperature and any unbound oligonucleotide removed by washing the beads with BW buffer. Total cellular RNA (40 μg), isolated from TGEV-infected cells, was made X6 SSC, 0.1% SDS (X1 SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), heated to 65°C for 3 min, cooled on ice, added to the magnetic beads/oligonucleotide complex and incubated at room temperature for 3 h. The RNA/magnetic bead complexes were washed with X6 SSC, 0.1% SDS and the TGEV [³H]-labelled RNA eluted with water at 65°C for 3 min and ethanol precipitated.

2.4. Determination of radioactivity in the mRNA species

Purified TGEV [³H]-labelled RNA was denatured with glyoxal and separated on 1% agarose gels as described by Sambrook et al. (1989). The gels were soaked in 0.8 M sodium salicylate (BDH) for 30 min to enhance the radioactive signal (Chamberlain, 1979), dried onto Whatman 3MM paper and exposed to pre-flashed X-ray film at -70°C for 4 days. The autoradiographs were aligned to the gels, using Glogos™ II (Stratagene) autoradiograph markers, to determine the position of the TGEV mRNA species, which were excised from the gel, fixed onto filtermat

A membranes (Wallac) and the amount of radioactivity determined by liquid scintillation counting on a LKB 1205 Betaplate counter. The number of counts for each mRNA species were corrected for the number of uridine residues, determined from sequence data (Britton and Page, 1990; Page et al., 1990; Britton et al., 1991), so that the amounts of radioactivity for each mRNA species corresponded to the molar ratios of the mRNAs.

3. Results

3.1. Purification of TGEV RNA

TGEV RNA, isolated using oligonucleotide B-153 linked to streptavidin magnetic beads, was eluted, denatured and analysed by agarose gel electrophoresis. Preliminary experiments had shown that 1 mg of the streptavidin magnetic beads bound 200 pmols of the biotinylated oligonucleotide and that the oligonucleotide/magnetic beads bound 98% of the ^3H -labelled RNA, indicating that virtually all of the TGEV RNA was removed from the RNA sample. The RNA profile (Fig. 1B) corresponded to that expected for TGEV (Page et al., 1990) confirming that the bound material was TGEV RNA. There was no evidence of ribosomal RNA, often observed as unlabelled ghost bands on glyoxal gels (Fig. 1A) which can alter the RNA profile. The background level of radioactivity, possibly resulting from incorporation of [^3H]uridine into cellular RNAs, breakdown products or incomplete TGEV transcripts, was greatly reduced in the purified RNA (Fig. 1). The results showed that this method gave purified TGEV mRNA suitable for a more accurate quantitative determination of the amounts of each TGEV mRNA.

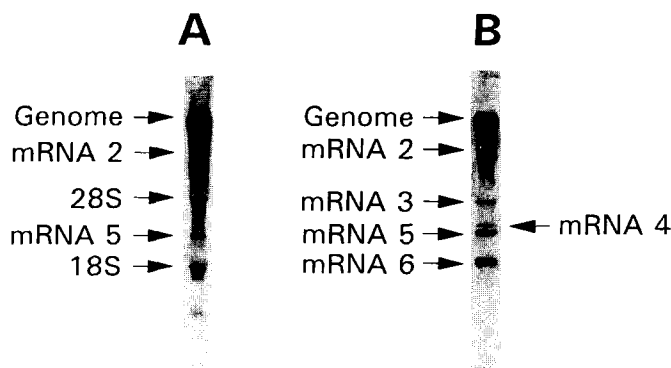


Fig. 1. Fluorograph of ^3H -labelled TGEV RNA following denaturation and separation by agarose gel electrophoresis. Lane (A) was from total cellular RNA and lane (B) following purification by the direct hybridization method. The RNA was hybridized to the magnetic bead/oligonucleotide complex for 160 min before elution. The gel was soaked in 0.8 M sodium salicylate, dried onto Whatman 3MM paper and autoradiographed.

3.2. Analysis of the amounts of TGEV mRNAs throughout a replication cycle

To investigate the production of TGEV mRNA species at early, middle and late time points in the replication cycle, infected cells were labelled with [³H]-uridine for 2 h periods, from 5 h to 13 h p.i. The mRNA species were purified using oligonucleotide B-153 linked to streptavidin magnetic beads and the amounts of radioactivity incorporated into each species over the time period was determined. Experiments were carried out three times. We were interested in investigating whether any of the TGEV mRNA species were preferentially synthesised at a particular time point of the replication cycle or whether they accumulated at a steadily increasing rate throughout the cycle. Most previous work on the characterization of TGEV mRNAs had only investigated the amounts of mRNAs produced 10 to 14 h p.i. using either metabolic labelling or Northern blot hybridization, both methods only indicating the final amounts after accumulation throughout the replication cycle.

All three experiments gave similar results so the mean values with standard deviations have been presented (Fig. 2). Our results showed that mRNA 6 was the

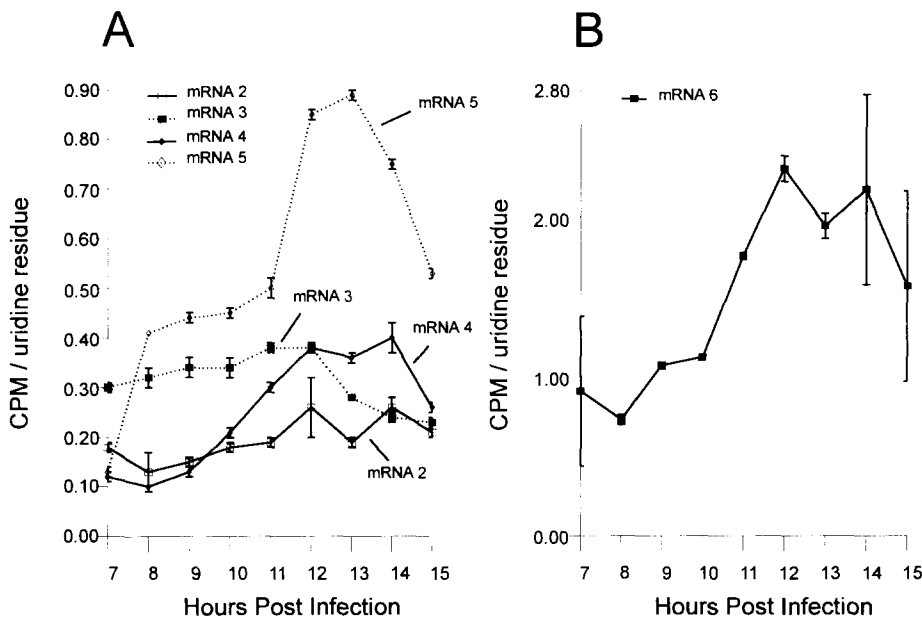


Fig. 2. Analysis of the amounts of the TGEV mRNAs produced throughout the replication cycle. At hourly intervals, from 5 to 13 h p.i., TGEV-infected LLC-PK₁ cells were labelled with [³H]-uridine for 2 h, total RNA was extracted and the TGEV RNA purified by the direct hybridization method. The subgenomic mRNAs were separated by denaturing agarose gel electrophoresis, excised from the gel and the amount of radioactivity measured. The counts were corrected for the number of uridine residues in each mRNA. The results obtained for mRNAs 2, 3, 4 and 5 are shown in (A) and for mRNA 6 in (B). The points represent the mean values, \bar{n} , for three experiments (n) and the bars represent the standard deviations calculated using σ_{n-1} .

most abundantly produced mRNA at all time points in the replication cycle. The amount, as measured from cpm/uridine residue, increased approximately three-fold from 7 h (representing incorporation of label 5 to 7 h p.i.) to 12 h p.i. (representing incorporation of label 10 to 12 h p.i.) (Fig. 2B) and the accumulation rate increased markedly after 10 h p.i. The amounts and rates of accumulation of the other subgenomic mRNAs during the 2 h labelling periods from 7 to 12 h p.i. varied depending on the species. The amount of mRNA 3 was about two-fold more than the other mRNAs (except mRNA 6) at 7 h p.i. but the amounts synthesised at the later time points only slightly increased and were observed to decrease after 12 h p.i. by about 50% (Fig. 2A). The amounts of mRNA 2 produced between 7 and 12 h p.i. only slightly increased, though the overall increase between the two time points was 1.4-fold (Fig. 2A). However, it should be noted that the amount of mRNA 2 was probably overestimated at all time points because of the co-migration of some degraded genomic RNA (Fig. 1). The rates and total amounts of mRNAs 4 and 5 produced 7 to 12 h p.i. followed a different pattern to the other mRNA species. The amounts of mRNA 4 produced increased 3.2-fold over the time period but did not appear to increase from the amount observed at 7 h p.i. until 10 h p.i., at which point the amount produced increased 1.6-fold and continued to increase until 14 h p.i. (Fig. 2A). The amounts of mRNA 5 produced increased 6.5-fold between 7 and 12 h p.i., consisting of a 3.2-fold increase at 7 and 8 h p.i., after which the rate of production remained almost constant until 11 h p.i., when there was a 1.8-fold increase between 11 and 12 h p.i. (Fig. 2A). No mRNA 7 was detected indicating that the amounts of this mRNA were below the levels of detection, though Northern blot analysis had detected low levels of mRNA 7 in previous studies (Jacobs et al., 1986; Page et al., 1990). The amounts of most of the mRNA species produced after 13 h p.i. were observed to decrease, indicating that the 12/13 h p.i. time point was the end of the replication cycle. The results indicated that all the TGEV mRNA species detected in this system were synthesised throughout the replication cycle, though mRNAs 5 and 6 appeared to be preferentially synthesised after 11 and 10 h p.i., respectively, and that mRNA 4 was observed to increase at 9 h p.i. Although production of most of the mRNA species increased in amounts throughout the cycle, which may be a reflection of the increase in amounts of genomic and anti-genomic RNA, the amounts of mRNA 3 surprisingly did not reflect this increase.

In addition we analysed the amounts of genomic RNA produced throughout the replication cycle. As we did not have complete sequence data for the TGEV polymerase gene, we calculated the number of uridine residues from a 1243 nt sequence of the polymerase gene that we had previously determined. The number of uridine residues formed about 33% of the total residues, a value similar to that determined for mRNA 2, indicating a total number of 9333 uridine residues for the genomic RNA assuming a genome size of 28 kb. The amounts of full-length genomic RNA in cpm/uridine residue ranged from 0.03 at 7 h, to 0.04 at 9 h and 0.05 at 12 h post infection, indicating that this was the least abundant RNA species and that the amounts produced appeared to slowly increase throughout the replication cycle. However, the amount of genomic RNA was underestimated due

to isolation of degraded genomic RNA ranging in size from about 8.3 kb (mRNA 2) to full length. Allowing for this the amounts of genomic RNA synthesised were probably in the region observed for mRNA 2, as observed by Sethna et al. (1989).

3.3. Correlation of the amounts of the TGEV mRNA species to the TASs

Previous work on MHV (Budzilowicz et al., 1985; Shieh et al., 1987) had proposed that the extent of base pairing between the TASs and the 3' end of the leader RNA controlled subgenomic mRNA abundance and provided an explanation of why MHV mRNA 7 was the most abundant subgenomic mRNA in MHV-infected cells. We compared the amounts of the TGEV mRNA species, produced throughout the replication cycle, with the degree of base pairing between the TASs and the 3' end of the leader RNA sequence to determine if there was any correlation with respect to the amounts of the mRNA species produced. The comparisons indicated that there were no obvious correlations between the base pairing and the amounts of mRNA synthesised. mRNA 6, the most abundant species in TGEV-infected cells, has a base pairing of 8 nts, whereas the least abundant mRNA, mRNA 7, undetectable in this system, has a base pairing of 10 nts. mRNAs 2 and 4 were observed to be produced in similar amounts early in infection, but later mRNA 4 increased more than mRNA 2, these mRNAs having base pairings of 7 and 6 nts, respectively. However, it could be argued that because mRNA 2 is twice the length of mRNA 4 that it would be expected to be produced in lower amounts, as observed for MHV.

4. Discussion

The aim of this study was to investigate the amounts of TGEV mRNAs produced throughout a replication cycle to determine (a) whether some mRNAs were synthesised earlier in the cycle than other species and whether the rate of accumulation of each mRNA was constant, giving rise to dissimilar amounts of the RNAs, and (b) to investigate whether there was any correlation between the amounts of the mRNAs produced and the degree of base pairing between the TAS sequences and the 3' end of the leader RNA. Analysis of the sequences preceding the TGEV genes indicated that there might be a correlation in the number of nts involved in base pairing between the 3' end of the leader RNA sequence and the TASs. However, it was also possible that some mRNA species were produced preferentially at different times of the virus replication cycle and that the analysis of the RNA at a late time point might have given a false impression of the amounts of the mRNAs. Thus a reproducible and efficient purification method allowing the accurate analysis of the amounts of the TGEV subgenomic mRNAs produced throughout the replication cycle was required.

We developed such a method that made use of a biotinylated TGEV-specific oligonucleotide in conjunction with streptavidin magnetic beads which resulted in the isolation of TGEV mRNAs free of ribosomal, other cellular RNAs and the

majority of potential breakdown products and incomplete transcripts derived from TGEV RNA. Previous attempts at the isolation of TGEV mRNAs using either poly-dT or oligo-dU column chromatography resulted in contamination with 28S and 18S ribosomal RNA potentially affecting their migration. Isolation of mRNA using oligo-dT or -dU chromatography required multiple purification steps to reduce the amounts of contaminating RNAs with resulting loss of mRNA. Multiple purification steps resulted in the purification of smaller mRNAs and the loss of the larger species during the washing steps (Ekenberg et al., 1992). The previous isolation methods also resulted in differential background levels of radioactivity, possibly resulting from incorporation of label into cellular RNA, incomplete TGEV RNA transcripts or from the breakdown of TGEV RNA and resulted in inaccuracies for quantitative determination.

Analysis of the amounts of the TGEV subgenomic mRNAs confirmed that they are differentially regulated. The results indicated that no particular mRNA species was produced for only part of the replication cycle. However, the amounts of some mRNA species increased significantly at various time points and led to the conclusion that there is some temporal control mechanism(s) regulating the production of the mRNAs. It was possible that mRNA 7 had been previously detected in low amounts at 10–14 h p.i. because it had been preferentially produced only early in infection, with subsequent degradation of the RNA. However, there was no evidence from our study to support this, unless mRNA 7 had been produced before 5 h p.i. and was then rapidly degraded. Surprisingly the data indicated that although mRNA 4 was initially produced in low amounts, similar to the levels observed for mRNA 2, the amount increased approximately two-fold later in the replication cycle.

The leader-priming model for coronavirus transcription relies on a negative sense copy of the genomic RNA from which the various mRNAs are transcribed, the amount of template for synthesis of the mRNAs being the same, theoretically allowing synthesis of equivalent quantities of each mRNA. In theory, the smaller a particular mRNA, the more product could be produced if the activity of the transcriptase is equivalent for each mRNA. The observation that some mRNAs are produced in different amounts and even produced in differential amounts during the replication cycle implies that there is some control over transcription. If the leader RNA recognises the TAS sequences via complementarity, the simplest control mechanism may result from different numbers of nts involved in base pairing between the leader RNA and the TAS. Analysis of the TGEV TASs identified different degrees of base pairing (Table 1). However, their comparison with the amounts of the mRNA species produced did not reveal any particular pattern.

Our observations indicated that if base pairing between the TAS and the leader RNA does play a role in controlling the synthesis of the mRNAs, it cannot be the only regulatory factor. Recent results on the analysis of the TASs for MHV mRNAs 3 and 7 by site directed mutagenesis, using a defective interfering (DI) RNA system (van der Most et al., 1994) indicated that the extent of base pairing between the leader RNA and the TAS did not control subgenomic RNA abun-

dance and that TAS recognition did not depend entirely on base pairing with the leader sequence.

If base pairing is not responsible or is only partially involved in the control of transcription, other control mechanisms have to be taken into account, e.g. the distance between the mRNA TAS and the initiation codon of the gene product (Table 1). Budzilowicz et al. (1985) hypothesized that the distance between the MHV TASs and the initiation codon of the gene product may also influence transcriptional regulation. The amount of a particular mRNA may be related to the amount or nature of the gene product, implying that the gene products could have negative or positive control over mRNA synthesis. For example, the most abundant coronavirus mRNA encodes the N protein, and in most cases, except for the TGEV group of coronaviruses, is the smallest mRNA produced. The protein is probably required throughout the replication cycle, to protect newly synthesized RNAs from degradation (Compton et al., 1987) and in the assembly of nucleocapsids with an additional increase in synthesis towards the end of the replication cycle to provide enough N protein for incorporation into progeny virus. In contrast, some of the least abundant coronavirus mRNAs potentially encode products that are produced in only small amounts. For example the gene product of TGEV mRNA 4, sM, has been detected on the cell surface at a late stage during the replication cycle with only 40 molecules per virion (Godet et al., 1992). This observation indicated that sM might only be required in small amounts late in the replication cycle consistent with the observed increase in synthesis of mRNA 4 after 9 h p.i. Interestingly the synthesis of mRNA 5 also increased late in the replication cycle and may be associated with the requirement of the M protein for incorporation into virions and for initiating budding of the nucleocapsids in the morphogenesis of the virus particles. Other control mechanisms may involve the degree of secondary structure, on the negative strand, around the TAS, which might control the availability of the TAS for interaction with the leader RNA and be involved in controlling the transcription. The observation that coronaviruses also produce negative copies of the mRNA species and that these have anti-leader sequences may imply that mRNAs may also be synthesised from the negative copies, either by leader-primed transcription or direct replication. The amounts of mRNA produced may then be regulated by the availability and/or amounts of the negative copies. Van der Most et al. (1994) indicated that the transcriptase complex might be able to recognise sequences upstream of the coronavirus genes without the involvement of base pairing implying that specific protein/nucleic acid interactions can take place and may control transcription.

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