Coupled Translation of the Respiratory Syncytial Virus M2 Open Reading Frames Requires Upstream Sequences*

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We have investigated the mechanism of the translation of the second open reading frame (ORF) of the respiratory syncytial virus M2 transcript that uses a novel coupled translation process requiring prior translation of the upstream ORF. The second M2-2 ORF sequences play no role in the coupling process and can be replaced with other gene sequences. Surprisingly, the overlap region of the two ORFs alone was not sufficient for coupled translation to occur. An analysis of the sequences required for the coupling process showed that portions of the transcript located along the length of the first ORF M2-1, upstream of the ORF overlap region, were essential for coupled translation to occur. A critically important region for this process was centered ~150 nucleotides upstream of the ORF2 initiation codons. This region was shown to contain a significant degree of secondary structure, and mutation of this sequence to remove predicted areas of base pairing significantly reduced coupled translation, confirming that the secondary structure was important for the coupling process. Additional sequences further upstream increased the efficiency of the coupled translation process. These data indicate that upstream sequences act in conjunction with the M2-1/M2-2 overlap region to promote coupled translation.

It has long been recognized that mRNA is a central molecule in the parasitic interaction of a virus with its host (1). Throughout an infection, viruses must rely on the translational apparatus of the host cell for expression of their genes. The study of translation of virus mRNA has identified many previously unknown processes used in their attempts to ensure selective translation of their own mRNA in the presence of large amounts of host mRNA. In addition, many viruses have been shown to encode more than one protein from an individual transcript, possibly in response to the pressure to maximize the coding capacity of the genome without generating additional energetic “strain” on the system that would arise by encoding a separate mRNA or because of other size/packaging constraints on the genome. Several of these strategies have subsequently been shown to be utilized by the host. Many of these novel processes are at the stage of initiation of translation. In the conventional translation mechanism, the initiation event involves the binding of the 40 S ribosomal subunit with eukaryotic initiation factors, GTP, and Met-tRNAi to a 5′-terminal cap structure on the mRNA followed by scanning movement in the 3′ direction (2). When the first AUG initiation codon is encountered, eukaryotic initiation factor 5B facilitates the binding of the 60 S ribosomal subunit and translation ensues following the release of all of the initiation factors and acquisition of elongation factors. If the sequence surrounding the first AUG is not favorable, the ribosome may scan past and initiate at the next AUG (3). Several viruses, most notably members of the family Paramyxoviridae, have utilized this action of leaky scanning to direct translation of additional protein(s) from a single mRNA. This is seen clearly in the case of Sendai virus for translation of the mRNA, which encodes the virus phosphoprotein. The open reading frame (ORF), which encodes the phosphoprotein, is initiated by an AUG codon in a suboptimal sequence context, and a proportion of the ribosomes, which have bound to the mRNA, continues scanning until they encounter the codon that directs synthesis of the C protein and which is in a good context for translation initiation. The same Sendai virus phosphoprotein mRNA also encodes two additional proteins, Y1 and Y2, by using a “ribosomal shunting” process in which the ribosome, instead of scanning in a progressive manner along the mRNA, is directed to the Y1 and Y2 initiation codons (4). A similar process has also been shown to be used by certain cellular mRNAs.

Picornaviruses, which lack the 5′-cap structure on their genomic/mRNA, direct translation initiation by virtue of the presence of a structural feature in the nucleic acid, the internal ribosome entry site (IRES) (5, 6). Ribosomes bind directly to the IRES and initiate translation at a downstream AUG codon. Since their discovery in picornaviruses, IRES have been studied in many other viruses and examples have been found within cellular transcripts (7). Pseudoknots are an additional example where a structural feature of viral mRNA directs translation of an alternative ORF. Here the secondary structures formed within the mRNA causes the ribosome to pause, which in some instances leads to a −1 frameshift to generate a protein with an alternative C terminus (8).

A novel process of translational control has been described for human respiratory syncytial virus (RSV) and the calicivirus rabbit hemorrhagic disease virus (RHDV). RSV is the major cause of hospital admissions of very young children for respiratory disease worldwide (9–11). RHDV is responsible for acute disease in wild rabbits and is closely related to human calici-

* This work was supported by the Biotechnology and Biological Sciences Research Council Grant 88/P16683. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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The abbreviations used are: ORF, open reading frame; RSV, respiratory syncytial virus; IRES, internal ribosome entry site; RHDV, rabbit hemorrhagic disease virus; EGFP, enhanced green fluorescent protein; CAT, chloramphenicol acetyltransferase; RT, reverse transcriptase; GFP, green fluorescent protein; RRL, rabbit reticulocyte lysate; ELISA, enzyme-linked immunosorbent assay.

Vol. 280, No. 23, Issue of June 10, pp. 21972–21980, 2005

Received for publication, March 1, 2005
Published, JBC Papers in Press, March 21, 2005, DOI 10.1074/jbc.M502276200

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Coupled Translation in the RSV M2 Transcript

Plasmid Construction—All of the PCR reactions were carried out using PfuTurbo Hotstart DNA polymerase (Stratagene, Amsterdam, The Netherlands), and resulting constructs were then sequenced. The sequences of primers used in this study (synthesized by TAG-Newcastle Ltd) are shown in Table I.

pWildEGFP—The enhanced green fluorescent protein (EGFP) ORF was amplified from pEGFP-N1 (Clontech) using the primers WildGFPF and WildGFPFR. This product was then cloned downstream of the RSV M2-1 ORF in-frame with the three internal start codons of M2-2 in the vector pBlueScript (Stratagene) generating the plasmid pWildGFPF (Fig. 1C).

EGFP-M2-CAT Fusion Mutants—The EGFP ORF was amplified from pEGFP using the primers GFP N terminus and GFP C terminus. Amplification with GFP C terminus removes the stop codon from EGFP and incorporates a SpII restriction site so that the overlap sequence from M2-1 ORF can be cloned in-frame and the M2-1 stop codon within the overlap is utilized. This PCR product was digested with KpnI and the CAT ORF was amplified (Fig. 2A) (17). Immediately downstream of the M2-1 stop codon, an XbaI site was used to fuse the CAT repressor site to anneal at designated points throughout M2-1. Amplification with GFP N terminus replaces the CAT ORF with the EGFP ORF cloned into the vector pBlueScript (Stratagene) for each plasmid in duplicate with the exception of pWildEGFP where 4-well replicates were used. The levels of CAT expressed from pWildCAT (set at 100%) was used to normalize expression to pWildCAT where 4-well replicates were used. The levels of CAT expressed from pWildCAT were normalized to pWildEGFP and then the optical readings were taken. Following a wash step, goat anti-rabbit HRP (Abcam, Cambridge, United Kingdom) diluted 1/16000 in blocking reagent was added for 1 h. A 1/10,000 dilution of Alab290 rabbit polyclonal anti-GFP primary antibody (Abcam, Cambridge, United Kingdom) was added for 1 h. A 1/1000 dilution of rabbit anti-HPR (Bio-Rad) (1/10,000 dilution reagent) then was added and incubated for 1 h. The plates were washed again, and ABTS substrate (Sigma) was added before measuring the optical density at 405 nm. Purified EGFP used for standards was obtained from Clontech.

RESULTS

Coupled Translation of Non-RSV Proteins—The construct pWildCAT shown in Fig. 1B contains the entire coding sequence of the M2-1 ORF (17). Immediately downstream of the M2-1 stop codon, an XbaI site was used to fuse the CAT reporter gene in-frame with the three start codons for the M2-2 ORF that were present upstream of the M2-1 stop codon. We have shown that the CAT reporter gene, which replaced the M2-2 coding sequence after the M2-1 stop codon, can be expressed in vivo from pWildCAT (17). To examine whether a protein other than CAT could replace the M2-2 ORF, we cloned the EGFP ORF in-frame with the three start codons of the M2-2 ORF in a manner identical to that of CAT gene (Fig. 1C). The EGFP gene was chosen, because it has a much higher G + C content (61.5%) than CAT (44.9%) and M2-2 (28.6%), which may affect expression, and the gene product is easy to assay in

The sequence of the overlap in each construct is shown (boxed). The stop codon of the first ORF is shown in boldface, the three start codons are underlined, and numbered 1–3, restriction enzyme sites SpII (GATC) and XbaI (TCTAGA) are underlined. A, wild type virus M2 ORFs cloned into plasmid pBS22K, B, pWildCAT. The RS virus M2-1 is the first ORF with the CAT ORF replacing the second viral ORF. The CAT ORF was shown to be expressed in vivo via the coupled mechanism (17). C, pWildEGFP. The EGFP ORF directly replaces the CAT ORF from pWildCAT. D, pC28. The M2 overlap sequence fused to the CAT ORF from pWildCAT was cloned downstream of the EGFP ORF via the SpII site (underlined left) surrounding the first ATG.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—All of the PCR reactions were carried out using PfuTurbo Hotstart DNA polymerase (Stratagene, Amsterdam, The Netherlands), and resulting constructs were then sequenced. The sequences of primers used in this study (synthesized by TAG-Newcastle Ltd) are shown in Table I.

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**pWildCAT Mutants**—The 5’ terminus of the M2-1 gene was amplified with primers T7 and WCSpH1a or T7 and WCSpH1b. These products were individually ligated to the PCR product generated using primers C28 and pBS-SphImut, which contains the remainder of the 3’ end of the M2-1 ORF and the CAT ORF, and inserted back into pBS.

**Primer Extensions and Toeprinting Assays**—Toeprinting assays were carried out as described by Gould et al. (19). Primer extensions were conducted by eliminating the in vitro transcription step of the toeprinting assay (19). Thus, the mRNA was synthesized in vitro and then the FAM-labeled primer was annealed to the template, which was immediately added to the reverse transcription reaction carried out at 25, 37, 42, or 45°C and analyzed using the GeneScan software as described previously (19).

**Transfection and Reporter Assays**—HEp-2 cells were transfected as described previously (17). The total amount of DNA added to each well was reduced to 250 ng, and the transfection reagent Lipofectin (Invitrogen) was used as recommended. CAT protein expression was measured using anti-CAT-coated plates and recommended components (Roche Applied Science) for each plasmid in duplicate with the exception of pWildCAT where 4-well replicates were used. The levels of CAT expressed from pWildCAT were normalized to pWildEGFP and then the optical readings were taken. Following a wash step, goat anti-rabbit HRP (Bio-Rad) (1/10,000 dilution reagent) then was added and incubated for 1 h. The plates were washed again, and ABTS substrate (Sigma) was added before measuring the optical density at 405 nm. Purified EGFP used for standards was obtained from Clontech.
a quantitative ELISA used in our laboratory. The data showed that expression levels of EGFP from pWildEGFP were similar to those of CAT from pWildCAT (data not shown). Thus, it appears that the sequence of the second ORF following the overlap region does not play a role in coupled expression.

To define the minimal parameters that enable coupled translation of the two M2 ORFs to occur, we attempted to co-express two heterologous proteins from the same mRNA transcript. To recreate the same physical arrangement as that seen in the RSV M2 transcript, the coding sequence for EGFP was inserted in place of the M2-1 ORF in pWildCAT, creating an EGFP/CAT dicistronic mRNA. However, the M2-1/M2-2 overlap region was retained so that the stop codon of the first ORF (EGFP) was positioned downstream of the start codons of the second ORF (CAT) constructing plasmid pC28 (Figs. 1D and 2A). The sequence of the overlapping region was identical to the M2 overlap region.

**FIG. 2.** A, construction of EGFP-M2-CAT fusions. Variable length segments from the 3' end of M2-1 (dotted box) and the CAT ORF (clear box) were amplified from pWildCAT using specific forward primers and the universal reverse primer pBSSphImut (Table I). The PCR products generated are shown. These were ligated via the SphI restriction site to the C terminus of EGFP that lacks its own stop codon so that the M2-1 stop codon is now utilized, as shown for pC28 in Fig. 1D. B, relative levels of CAT protein expressed from the engineered constructs presented as a percentage of the positive control pWildCAT. The negative control pT7GFP expressing only the GFP ORF from a T7 promoter is shown. C, top, sequence alignment of selected segments of pC160, pC154, pC148, and pC142. The deletion of 6 nucleotides from pC148 to pC142 is enough to abolish CAT expression. Bottom, pWildCAT SphI mutations.

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lapse with the exception of one mutation around AUG1 where the SpH I site had been introduced for cloning purposes (plasmid pC28, Fig. 1D). pC28 was transfected into vaccinia vTF7.3-infected HEp2 cells and the expression of CAT protein was measured (Fig. 2B). Fig. 2B shows that no CAT could be detected from pC28. The transfection and expression system was functioning as the product of the first ORF, EGFP, was detected (data not shown). Furthermore, the positive control pC580, which contained EGFP fused to almost all of the M2-1 ORF with the exception of the first four nucleotides from the 5’ end, when transfected into cells expressed CAT protein to a level similar as that measured in pWildCAT (Fig. 2, A and B).

To ensure that introducing a mutation within the overlap sequence in pC28 (Fig. 1D) by inserting the SpH I site around the first start codon was not interfering with the coupled mechanism, the plasmid pC79 was constructed (Fig. 2A). pC79 contained 79 nucleotides from the 3’ end of the M2-1 ORF fused in between the EGFP and CAT ORFs, and in this plasmid, the sequences in the immediate vicinity of the overlap region are the same as those in the M2 mRNA. Quantifying the levels of expression of CAT from pC79 also enabled the role of the 47 nucleotides directly upstream of the overlap to be investigated. Unexpectedly, even with these additional sequences, it was still not possible to detect coupled translation of CAT protein, although EGFP was produced (Fig. 2B). Therefore, the sequence representing the last 79 nucleotides of M2-1 were not sufficient alone to couple translation even with a fully conserved overlap sequence. These data together with the observation that coupled translation of EGFP and CAT ORFs does occur if the entire M2-1 ORF is fused in the correct frame between the two ORFs (pC580 Fig. 2B) indicate that sequences in the main body of the M2-1 ORF are required for coupled translation.

A series of additional plasmids were constructed in a systematic manner to contain increasing lengths of sequence from the 3’ end of M2-1 ORF inserted between the EGFP and CAT ORFs to determine the minimal length of sequence required for coupled translation (Fig. 2A). In particular, we focused in detail using 6-nucleotide increments on an area of the M2-1 ORF 142–160 nucleotides upstream from the stop codon (Fig. 2C). Each of these plasmids was transfected into HEp2 cells infected with vaccinia vTF7.3, and CAT protein expression was measured (Fig. 2B). The data showed that, to obtain a detectable level of CAT protein, at least 148 nucleotides from the 3’ end of the M2-1 ORF were required (plasmid pC148). CAT expression could not be detected in the construct pC142, which contained just 6 nucleotides (CCATCT) less than pC148. Indeed, the SpH I site used in the cloning process matched 3′ of the 6 nucleotides absent in pC142 (Fig. 2C). A significant rise in CAT expression was also noted between the mutants pC181 and pC202 (Fig. 2A and B). The levels of CAT expression then began to plateau between constructs pC202 and pC484 with a further small increase seen between pC484 and pC532. Whereas the difference in expression between pC484 and pC532 is not statistically significant, the consistently observed rise may suggest that this region has a minor role in coupled translation.

The level of EGFP expression was also measured from each construct in an ELISA. This showed that there was an apparent decrease in the amounts of EGFP detected, which correlated with the length of M2-1 gene present (data not shown). This is most likely to be the case because, as the fusion protein increases in size with longer lengths of M2-1 fused to EGFP, the ELISA is no longer detecting the proteins with equal efficiency, giving an untrue impression of a reduction in protein production from ORF1.

The effect of replacing the 6 nucleotides shown to be required for coupled translation in the EGFP-M2-CAT constructs with the SpH I site in pWildCAT (Fig. 2C, pWildCAT-SA) was investigated. An additional mutant was also constructed in which the SpH I site was inserted as additional 6 nucleotides within this region (Fig. 2C, pWildCAT-SB). CAT expression was measured and showed that either the direct replacement or the insertion of the SpH I restriction site in pWildCAT at this position caused a significant decrease in coupled expression (Fig. 2B), suggesting an important role for this region of the M2-1 ORF in the coupled translation process.

**M2-1 RNA Analysis—**To investigate the mechanism(s) that leads to the ribosomes re-initiating at the second ORF, we carried out a primer extension inhibition (toeprinting) assay that could be used to determine the position of ribosomes along mRNA (20–22). Toeprinting has been used previously to study the interactions of ribosomal components on IRES sequences (23, 24). The assay works by translating mRNA in vitro with rabbit reticulocyte lysates (RRLs). When cycloheximide is added to the reaction, elongation is blocked, marking the position of the ribosomes on the transcript. The mRNA/RRL complex is then copied into cDNA using a labeled primer, and where the reverse transcriptase (RT) meets the ribosome bound to the mRNA, polymerization is halted, generating a fragment of a designated size. If the positioning of ribosomes is not random and instead the ribosomes are clustered at a specific point on the mRNA, a “toeprint” fragment is generated. The two M2 ORFs were first synthesized in vitro using T7 RNA polymerase by linearizing plasmid pRS222K with HindIII (Fig. 1A). An mRNA fragment of 956 nucleotides representing the full-length transcript was obtained (Fig. 3, lane 2). However, an additional band of 562 nucleotides was always seen. This second band represents premature termination of the T7 polymerase within the M2-1 ORF, because transcription of pRS222K linearized with NdeI, which cuts upstream of the HindIII site in the M2-2 ORF, still results in the 562-nucleotide band (Fig. 3, lane 3). Premature termination was again seen when the M2-1 ORF was re-cloned downstream of the bacterio-oph age T3 promoter in pBC(SK+) (Stratagene) (Fig. 3, lane 4). However, when plasmid pRS222K was linearized with KpnI and RNA was synthesized with the T3 RNA polymerase (which uses the coding strand as the template), there was no evidence for premature termination (Fig. 3, lane 5). It was possible to use the mixed mRNA species in the toeprinting assay, because the primer does not anneal to the smaller fragment. However,
the significance of the presence of the prematurely terminated transcription product is discussed below.

The mRNA synthesized in vitro from pRS22K digested with HindIII was used in a toeprinting assay. The primer M2 toe1 (Table I) was annealed to the mRNA and added to in vitro translation reaction mixture with RRL. Areas where the ribosomes paused on the mRNA transcript would be represented as peaks on the resulting electropherogram, because further progression of the RT is inhibited (19). As a control, the same mRNA/primer mixture was added to the translation reaction mixture but with no RRL present. This control indicates the regions within the mRNA that causes the RT polymerase to pause, presumably due to regions of strong secondary structure. Identical electropherograms were seen in the presence or absence of RRL in a toeprinting assay with the RT step carried out at 37 °C (Fig. 4A). The traces showed the distinctive pattern of a series of peaks with one region having a broad shape that slopes away from an initial pinnacle. A sharper peak of 201 nucleotides also appeared. The intensity of this peak varied between repeat experiments. Because these peaks are present when no RRLs are added, they must represent regions in the RNA strong in secondary structure that cause the RT polymerase to pause or fall from the transcript, even in the absence of ribosomes. The sizes of the peaks were determined based on the ROX size standards added to each reaction (orange peaks, Fig. 4). However, it is known that the ROX standards underscore the fragments by 2–3 nucleotides so the traces were aligned with a sequencing ladder generated using the same primer (19). When the sequencing reactions were assembled, indistinguishable peaks were found (data not shown). The absence of the broad peak was present. Interestingly, when mRNA was synthesized in vitro from these plasmids, the smaller fragment representing premature termination was only found in pC580 and pC148 (Fig. 3, lanes 7–10). Because the second ORF in these constructs was the CAT ORF and not M2-2, the primer CAT2 (Table I) was used for the RT reaction. The position of the broad peak was 329 nucleotides from the 5′ end of CAT2 as seen for pC580 (Fig. 5A). However, the broad peak was not present in pC142, which also cannot couple translation. The 3′ end of the broad peak differs by 1 nucleotide between pC148 and pC580 and the other mutants tested falling between these two points in a pattern that is dependent on the length of M2-1 sequence present (data not shown). The absence of the broad peak in pC142 is predicted to be because the 6 nucleotides missing here, but present in pC148, are an essential component in the formation of any secondary structure.

Further evidence supporting this was shown when the primer extension assays were carried out on the pWildCAT mutants generated that have the SphI restriction site inserted within this region (shown in Fig. 2C). Only mRNA synthesized from pWildCAT contained the premature termination fragment (Fig. 3, lanes 12–14) and generates the broad peak seen in the assay (Fig. 5B).

### DISCUSSION

Translation of the two ORFs in the RSV M2 mRNA has been shown to be coupled, with translation of the downstream, M2-2, ORF dependent on completion of translation of the upstream, M2-1, ORF (17). Translation of the second ORF of the RSV M2 transcript is not dependent on the presence of an IRES sequence within M2-1, because it has previously been shown that translational termination at the first ORF is an essential for translation of the second ORF (17). Coupled expression of three different ORFs, M2-2, CAT (14, 17), and EGFP, cloned downstream of the M2-1 ORF, has now been measured. These data show that the sequence of the second ORF after the M2-1 stop codon can be changed; therefore, the downstream region plays no direct role in the translational coupling process. It is noteworthy that the G+C content of the EGFP ORF contrasts starkly with that of the viral M2-2 ORF, indicating that this is also not a factor in the coupling process.
A, overlapping toeprinting electropherograms of M2 mRNA from reactions with either RRL present (red) or absent (gray) using the primer M2toe1 for the primer extension step. The characteristic broad peak indicating a region of stable secondary structure is labeled. ROX size standards are shown in orange. B, overlay of the M2 mRNA/no RRL reaction trace shown in Fig. 4A (gray) with the sequencing ladder generated using M2toe1 (green, ddATP; blue, ddCTP; black, ddGTP; and red, ddTTP).
Surprisingly, it was not possible to couple the expression of two heterologous ORFs encoding EGFP and CAT with just the M2 overlap sequence alone joining the two ORFs. To investigate whether additional sequences within the M2-1 ORF, together with the overlap region, were essential for coupled translation, a series of constructs were made that progressively
increased the length of the M2-1 sequence between the EGFP and CAT ORFs. Expression from these constructs showed that at least 148 nucleotides from the 3' end of M2-1 were essential for coupled translation to occur at all. However, even with these 148 nucleotides present, the levels of expression from the second ORF were reduced by 10-fold compared with those seen when the entire M2-1 ORF was present upstream of the overlap region, reflecting the situation in the normal M2 mRNA (Fig. 2B). The presence of 256 nucleotides from the 3' terminus of the M2-1 ORF increased coupled translation levels to 82% of the "wild type." Wild type levels of coupled translation were only achieved when 532 nucleotides of the M2-1 ORF were present. Thus, the majority of M2-1 ORF participates in the process of coupled translation, with some segments more important than others. This differs significantly from the findings for the RHDV calicivirus subgenomic mRNA where only 84 nucleotides of the sequence upstream of the overlap region recovered ~90% wild type expression levels of ORF2 (18).

Following the original description of the coupling process, several mechanisms were proposed involving the possibility of ribosomes pausing within the overlap region so that a backward movement of the ribosome onto the start codon(s) of the second ORFs could occur (17). Toeprinting assays carried out to determine the positions of ribosomes stalled along the M2-1 transcript did not detect evidence of an accumulation of stalled ribosomes within the overlap region. A striking feature of the toeprinting electropherograms was the presence of a broad peak with a 3' position located 137 nucleotides from the stop codon of M2-1. The characteristic shape seen with this peak represents an area of ~22 nucleotides. No differences were found in the electropherogram patterns in the presence or absence of ribosomes (Fig. 4A). Thus, the major peak seen represents the position where the elongation of the reverse transcriptase is inhibited by the intrinsic proprieties of the mRNA itself. These data indicate that there is a very high degree of secondary structure in a specific region within the M2 transcript where the RT pauses on or falls from the RNA template. cDNA synthesis can occasionally continue beyond this point in some individual reactions, because additional peaks were seen when samples were overloaded (data not shown). False priming was eliminated, because the main peaks were also seen using a different primer (Fig. 5). The same regions were also identified during the dideoxynucleotide sequencing reactions as being areas where extension with the polymerase was unable to process (Fig. 4B). The 3' end of the broad peak was located in close proximity to the sequence shown by the EGFP-M2-CAT fusion analysis to be the minimal requirement for coupled translation to occur. A strong correlation can be seen between the presence of the broad peak in the toeprinting assay and coupled translation. For example, the peak was found with plasmid pC148 that can express CAT but was absent in plasmid pC142, which does not couple translation. However, it should be noted that, in these constructs, no additional upstream sequences from the M2-1 ORF were present.

When M2 mRNA was synthesized in vitro using T7 RNA polymerase, a shorter transcript representing premature termination was always observed. This was most likely due to the region of stable secondary structure generating a prematurely terminated mRNA transcript. In contrast, with constructs lacking the region or structure, only a single mRNA transcript of the expected size was produced. While confirming the presence of a region of stable secondary structure, this raises questions regarding the efficiency of the RNA synthesis in the M2 gene during transcription and replication from the virus genome. When the M2 gene was cloned in the reverse orientation so that the coding stand was the template, premature termination did not occur. This would be analogous to the single-stranded antigenome template where premature termination would not be desirable.

The program Mfold (25) was used to generate a predicted structure of the mRNA in the vicinity of the region of strong secondary structure (Fig. 6). The program predicted a series of stem-loop structures in this region. The truncated M2-1 segment in plasmid pC142 would not be able to form this structure, because a significant proportion of the RNA sequence involved its formation is absent. The mutation of the secondary structure region to generate mutants pWildCAT-SA and pWildCAT-SB prevented the formation of the broad peak, representing the region of secondary structure, in the toeprinting assay (Fig. 5B). This is because the mutations in pWildCAT-SA would reduce the stability of the predicted stems I and III (Fig. 6) by eliminating the formation of three base pairings. In the case of pWildCAT-SB, an additional 6 nucleotides were inserted into stem I. However, coupled translation can still occur in these mutants, albeit at a significantly lower level than pWildCAT (Fig. 2B). The low level of coupled expression with these mutants is predicted to occur, because there may be partial destabilization of the structure and/or unlike the construct pC142 that also lacks the structured region. These constructs contain the full-length wild type upstream M2-1 sequences that have been shown in this study to also play an important role in the coupling process. The differences in the level of coupled translation seen with between pWildCAT-SA and pWildCAT-SB may be due to the different number of stems disrupted. Taken together, these data demonstrate the critical importance of the secondary structure in the ability of the RNA to couple translation of the two ORFs. However, in interpreting these data, it should be borne in mind the 3' terminus of the broad peak seen in the toeprinting assay was positioned within the predicted structure shown in Fig. 6. The reason for this is not clear, but it may indicate that the structural element involved is more complex than that generated by modeling.

In conclusion, we have shown that the process of coupled
translation of the M2 mRNA of RSV occurs by a novel mechanism. The data presented here indicate that the mechanism of the coupled translation process for the RSV M2 mRNA is not the result of a simple scanning by ribosomes after completion of translation of the upstream M2-1 ORF but requires structural features present in the mRNA. We have identified three sections of the M2-1 transcript that play a role in the coupling process: 1) the overlap sequence (17); 2) the region of ~22 nucleotides in length and strong in secondary structure centered 150 nucleotides from the M2-1 stop codon; and 3) a larger less well defined region upstream that may extend to the start codon. In particular, the ability to couple translation is strongly associated with the region of strong secondary structure upstream of the overlap region, which also appears to affect \textit{in vitro} transcription reactions. Further work is required to decipher the precise nature of the potential molecular interaction(s) in the upstream ORF, particularly between the sequences containing the secondary structure and the overlap sequence.

Acknowledgments—We thank Dr Helen Bird and Lesley Ward for technical assistance in the toeprinting assays, Rowan Gibson for the construction of pC28, Oliver Dibben for designing the EGFP ELISA, and Dr. Tony Marriott for comments on the manuscript and pT7GFP.

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J. Biol. Chem. 2005, 280:21972-21980.
doi: 10.1074/jbc.M502276200 originally published online March 21, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M502276200

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