Leaky Scanning and Scanning-independent Ribosome Migration on the Tricistronic S1 mRNA of Avian Reovirus*

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The S1 genome segments of avian and Nelson Bay reovirus encode tricistronic mRNAs containing three sequential partially overlapping open reading frames (ORFs). The translation start site of the 3′-proximal ORF encoding the σ C protein lies downstream of two ORFs encoding the unrelated p10 and p17 proteins and more than 600 nucleotides distal from the 5′-end of the mRNA. It is unclear how translation of this remarkable tricistronic mRNA is regulated. We now show that the p10 and p17 ORFs are coordinately expressed by leaky scanning. Translation initiation events at these 5′-proximal ORFs, however, have little to no effect on translation of the 3′-proximal σ C ORF. Northern blotting, insertion of upstream stop codons or optimized translation start site, 5′-truncation analysis, and poliovirus 2A protease-mediated cleavage of eIF4G indicated σ C translation derives from a full-length tricistronic mRNA using a mechanism that is eIF4G-dependent but leaky scanning- and translation reinitiation-independent. Further analysis of artificial bicistronic mRNAs failed to provide any evidence that σ C translation derives from an internal ribosome entry site. Additional features of the S1 mRNA and the mechanism of σ C translation also differ from current models of ribosomal shunting. Translation of the tricistronic reovirus S1 mRNA, therefore, is dependent both on leaky scanning and on a novel scanning-independent mechanism that allows translation initiation complexes to efficiently bypass two functional upstream ORFs.

The scanning model of translation initiation accounts for the vast majority of translation events occurring in eukaryotic cells (1). Recruitment of the 40 S ribosomal subunit is facilitated by the presence of a 5′-terminal m7Gppp cap and canonical eukaryotic initiation factors (eIFs) involved in cap binding and creation of the 43 S and 48 S preinitiation complexes. Translation initiation generally occurs at the 5′-proximal AUG start codon after scanning of the 5′-untranslated region (UTR) by the preinitiation complex and recruitment of the 60 S subunit. Alternate sites of translation initiation, however, almost certainly contribute to the complexity of both viral and cellular proteomes. For example, the sequence context flanking an AUG start codon contributes to the efficiency of translation initiation, with the optimal context being GCCRCCAUGG; the purine in position −3 confers the greatest enhancement on initiation followed by the G at position +4, particularly in the absence of A at position −3 (2). Scanning ribosomes may scan past a suboptimal 5′-proximal AUG codon, initiating translation at alternate downstream methionine codons.

In addition to leaky scanning, three additional mechanisms of alternate translation initiation have been described (3). After translation of a short upstream ORF (uORF), 40 S subunits may resume scanning and reinitiate translation at a downstream ORF (4). Reinitiation is generally inefficient and is favored by translation of a short uORF (~2–50 codons), which may prevent the time-dependent dissociation of essential initiation factors, and by an intercistronic distance of >70 nucleotides that may allow sufficient time for scanning 40 S subunits to reacquire the eIF2-GTP-Met-tRNAi cofactor required for translation initiation (5, 6). Internal ribosome entry site (IRES) elements are complex secondary structures that facilitate direct recruitment of the preinitiation complex bypassing cap-dependent scanning to access internal AUG start codons (7). The third mechanism, ribosomal shunting, is extremely rare and involves the non-linear 5′-3′ transfer of scanning ribosomes, bypassing the intervening region to access an internal start codon (8). Shunting has only been described for a few viral, and possibly cellular, mRNAs (9–12).

The S1 mRNAs transcribed by the fusogenic avian (ARV) and Nelson Bay (NVB) reoviruses encode three unrelated proteins from sequential, partially overlapping ORFs (Fig. 1A) (13, 14). The tricistronic gene arrangement of the ARV and NVB S1 mRNAs has few parallels in the eukaryotic world, and the mechanism(s) that regulates translation of these remarkable S1 mRNAs remains largely unexplored. The 5′-proximal ORFs of the ARV and NVB S1 mRNAs, which are initiated by AUG codons in a suboptimal context (Fig. 1B), encode the p10 fusion-associated small transmembrane proteins responsible for the syncytium-inducing phenotype of these fusogenic reoviruses (15). The p17 protein encoded by the second ORF is a nonstructural nucleocyttoplasmic protein of no known function (16). With an A residue at position −3, the most conserved and

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4The abbreviations used are: eIF, eukaryotic initiation factor; UTR, 5′-untranslated region; ORF, open reading frame; uORF, upstream ORF; IRES, internal ribosome entry site; ARV, avian reovirus; NVB, Nelson Bay reovirus; EGFP, enhanced green fluorescent protein; RT, reverse transcription; qRT, quantitative RT.

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functionally most important flanking residue, the ARV p17 start codon is in a strong context for translation initiation. The 3′-proximal ORF encodes the σC protein, a soluble cytoplasmic protein structurally and functionally related to the non-fusogenic mammalian reovirus σ1 cell adhesion protein (17, 18). The start codon of the σC ORF exists in a preferred context, but it occurs 630 nucleotides downstream of the 5′-terminal cap structure (Fig. 1). This long leader sequence contains four AUG codons, at least two of which are functional start codons for the p10 and p17 ORFs and at least one of which exists in a strong context for an initiator methionine (Fig. 1B). How ribosomes access the σC start site is unknown.

Based on the arrangement of ORFs and the absence of any methionine codons in the intergenic region between the p10 and σC ORFs, translation reinitiation was posited as a possible mechanism for σC expression (1). According to this scenario, the p10 ORF would provide access to the σC start codon by “escorting” some ribosomes past the strong p17 start site. After translation termination of the p10 ORF, 40 S subunits would remain attached to the mRNA and resume scanning to reinitiate translation at the downstream σC start site.

Two additional features of σC expression, however, appear contradictory to a translation reinitiation mechanism. First, the 98-codon p10 ORF is considerably larger than what would be expected for an uORF that directs translation reinitiation (6). Second, optimizing the context of the p10 start site results in enhanced translation of the p10 ORF with no apparent qualitative increase in σC expression (14). In view of these considerations, two alternative mechanisms for translation initiation at the internal σC start codon have been posited, IRES-mediated translation or ribosomal shunting (14, 19). However, the extremely short 5′-UTR present in the S1 mRNAs (i.e. 24–26 nucleotides) is inconsistent with existing models of IRES- or shunting-mediated translation initiation.

The absence of any detailed expression analyses of the tricistronic ARV or NBV S1 mRNAs precludes any meaningful speculation on which alternative translation initiation mechanisms might be operative. To address this issue, we undertook a rigorous quantitative analysis of ARV S1 mRNA translation. We now provide direct evidence that translation of the p10 and p17 ORFs is coordinately regulated by context-dependent leaky scanning. Contrary to expectations, however, the two most common mechanisms for translation initiation at internal start codons, translation reinitiation or IRES elements, do not contribute to σC translation. Further analyses revealed that a novel eIF4G-dependent, scanning-independent mechanism provides efficient ribosome access to the internal σC start codon in a manner that is relatively unaffected by translation initiation events occurring at either of the two functional upstream ORFs.

FIGURE 1. Gene arrangement of the ARV and NBV S1 genome segments. A, the organization and arrangement of the p10, p17, and σC cistrons (shaded rectangles) contained within the ARV and NBV S1 genome segments are depicted. Numbers refer to nucleotide positions of the genome segment, and the first and last positions of the ORFs (excluding the termination codon). B, the locations and flanking sequences of all potential initiator methionine codons (underlined) in the S1 cDNA sequences of the ARV and NBV S1 genome segments upstream of the σC start site are listed. The codons are identified by the first nucleotide position of the methionine codon. The reading frame of each methionine codon is indicated (parentheses). The Met-326 codon in NBV occurs out-of-frame in the p17 ORF.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—The quail fibroblast cell line, QM5, was maintained as previously described (20) and used for all transfection studies. Rabbit antisera that recognize σC, p10, or p17 were previously described (14). Rabbit antisera specific for the full-length ARV p10 protein or the endodomain of the ARV p10 protein were prepared as previously reported (15). Additional rabbit antisera included sera specific for actin (Sigma) enhanced green fluorescent protein (EGFP) (Clontech) or human eIF4G (a gift from N. Sonenberg, McGill University). Horse serum and conjugated goat anti-rabbit (KPL, Gaithersburg, MD) or anti-mouse (Jackson Immunoresearch Laboratories) immunoglobulin was used as a secondary for Western blots.

Plasmids—Plasmids pARV-S1 and pNBV-S1, containing the full-length cDNAs of the S1 genome segments of ARV or NBV cloned into the HindIII-NotI sites of pcDNA3, were previously described (15). The pARV-S1 expression plasmid was point-substituted using the QuickChange site-directed mutagenesis kit, as per the manufacturer’s instructions (Stratagene) to optimize the context (CCACCAUGG) of the p10 or p17 start codons (plasmids p10opt and p17opt), to insert stop codons in the p10 ORF (plasmids p10stop84 and p10stop243), or to delete four start codons that occur upstream of the σC start site (plasmid pARV-S1mono). Specific primers were used to generate 5′-terminal truncations of pARV-S1 (plasmids pARV-S1d147, -d303). The poliovirus 2A protease gene was subcloned from pPV802 (provided by A. Kaminski, Cambridge University) into pcDNA3 using restriction sites HindIII and EcoRI to generate pPV2A. The full-length ARV and NBV S1 cDNAs from the pARV-S1 and pNBV-S1 vectors were subcloned downstream of the EGFP ORF in plasmid pEGFP-N1 (Clontech) using the NotI restriction site. The poliovirus type 2 Lansing strain IRES was inserted between the full-length S1 mRNA and the EGFP ORF in the pEGFP-N1 backbone. All expression constructs were confirmed by sequencing.

In Vitro Transcription and Translation—Capped transcripts were synthesized in vitro using T7 RNA polymerase and NotI-linearized pARV-S1, p10opt, or p17opt plasmids as templates, as previously described (14). The quality of the mRNA was assessed using denaturing agarose gels and ethidium bromide staining. The mRNA (250 ng) was translated in rabbit reticulo-
cyte lysates (Promega) in the presence of 1 μCi/50-μl reaction of [3H]leucine, as specified by the manufacturer.

Immunoprecipitation Analysis—Q5 cells transfected with the various expression plasmids were pulse-labeled with 50 μCi/ml of [35S]methionine or [3H]leucine for 30–60 min at 24–36 h post-transfection, and radiolabeled p10 or σC was detected by immunoprecipitation and SDS-PAGE as previously described (15). Expression levels were determined by quantifying the pixels present in specific polypeptide bands as described below. Control titration experiments determined that antibody levels were saturating in the immunoprecipitations, and different exposures of the fluorograms were used to ensure a linear exposure range.

Co-transfection with the Poliovirus 2A Protease—Q5 cells were co-transfected with pPV2A and the indicated expression plasmids using Lipofectamine (Invitrogen) and equal concentrations of each plasmid DNA. Cells were fixed and immunostained with the antiserum recognizing σC to reveal the presence of antigen-positive syncytial foci, as previously described (15). Fluorescent images of live cells transfected with pEGFP were captured at 200× magnification. Alternatively, lysates of co-transfected cells were used for quantitative Western blotting as described below.

Northern Blotting—Total RNA was extracted from transfected cells using Trizol (Invitrogen) according to the manufacturer’s specifications. Total RNA (8 μg) was fractionated on formaldehyde gels and stained with ethidium bromide to reveal the 28 S and 18 S ribosomal RNA markers. The gels were blotted onto Hybond membranes (Amersham Biosciences) and probed with [32P]GTP-labeled S1-specific probes generated using random hexamers and the gel-purified, full-length S1 genome segment cDNAs as templates. Bound probe was detected by autoradiography with intensifying screens.

Quantitative Western Blotting—Cell lysates in decreasing 1-μg increments (i.e. 6, 5, 4, or 3 μg of cell lysate) were fractionated by SDS-PAGE (15% polyacrylamide) and transferred to Hybond membranes (Amersham Biosciences) and probed with [35S]methionine or [3H]leucine for 30–60 min at 24–36 h post-transfection, and radiolabeled p10 or σC was detected by immunoprecipitation and SDS-PAGE as previously described (15). Expression levels were determined by quantifying the pixels present in specific polypeptide bands as described below. Control titration experiments determined that antibody levels were saturating in the immunoprecipitations, and different exposures of the fluorograms were used to ensure a linear exposure range.

RESULTS

Efficient σC Translation Initiation Occurs from a Full-length Tricistronic S1 Transcript—Cryptic splice sites or promoters or mRNA cleavage upstream of an internal start codon can potentially confound translation analysis of polycistronic mRNAs (7, 22). To exclude such trivial explanations for σC translation initiation, S1-specific transcripts generated in cells transfected with either the pARV-S1 or pNBV-S1 expression constructs were detected by Northern blotting. In both cases only a single species of S1-specific mRNA was detected, and these transcripts migrated slightly ahead of the ~1850-nucleotide 18 S rRNA species, as expected for the full-length S1 transcript (Fig. 2A). Therefore, σC translation derives from a full-length, tricistronic S1 mRNA, not from a truncated mRNA containing the σC start site as the 5’-proximal AUG codon.

In general, uORFs exert a strong negative effect on translation initiation at downstream ORFs (1, 6, 23). To obtain an estimate of how efficiently ribosomes access the internal σC start site, the four AUG codons that occur upstream of the σC ORF were removed by site-directed mutagenesis to generate pARV-S1mono. The transcript encoded by this plasmid is monocistronic, with the σC start site representing the 5’-proximal start codon. Quantitative analysis from two independent experiments indicated a 1.7- and 2.9-fold increase in σC expression levels from this monocistronic mRNA relative to σC translation from the polycistronic S1 mRNA (Fig. 2B). This difference, however, was not statistically significant, suggesting the uORFs exert little to no effect on ribosome access to the σC start site. By comparison, removal of the uORFs present in the yeast GCN4 or mouse ATF4 mRNAs, two examples of mRNAs where translation is regulated by reinitiation, stimulates translation of the major downstream ORFs by 74–500-fold (4, 24). The Northern blotting and monocistronic S1 mRNA results suggested that the mechanism responsible for σC translation initiation functions almost as efficiently as linear scanning and in a manner that is relatively unaffected by the presence of the uORFs to provide ribosome access to the internal σC start codon present on a full-length, tricistronic mRNA.

Coordinated Expression of the p10 and p17 ORFs by Leaky Scanning—To further explore any interrelationship between translation initiation events occurring at the uORFs and the σC ORF, the suboptimal p10 and p17 start codons (p10, CGUC-GAUGC; p17, GCACAAGUGC) were individually converted to

Avian Reovirus Tricistronic S1 mRNA

qRT-PCR—Total cellular RNA was obtained from transfected Q5 cells using Trizol (Invitrogen) and the RNeasy kit (Qiagen). cDNA produced through reverse transcription from 0.25 and 0.5 μg of the purified RNA was then subjected to real time PCR utilizing the SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green (Invitrogen), a MX3000p Thermocycler (Stratagene), and an amplification protocol consisting of a 10-min hot start at 95 °C followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 1 min, and elongation at 72 °C for 30 s. Melting curves as well as separation of PCR products on a 1% Tris acetate-EDTA agarose gel were used to ensure the formation of a single product of the appropriate size. Relative gene expression normalized to β-actin expression was calculated using the ΔΔCT method (21).
Avian Reovirus Tricistronic S1 mRNA

an optimal context (CCACCAUGG) to generate the expression constructs p10opt and p17opt. Reticulocyte lysates were primed with the capped, in vitro transcribed mRNAs derived from the parental pARV-S1 construct or the optimized constructs, and the [3H]leucine-labeled translation products were detected by SDS-PAGE and fluorography. Qualitative analysis of the total translation mixtures and of immunoprecipitated lysates revealed αC translation from all three mRNAs (Fig. 3). Radiolabeled, low molecular mass (<17 kDa) translation products were also present in the total translation mixtures (Fig. 3, lanes 1–3), which were poorly resolved due to comigration with the large quantities of hemin present in the reticulocyte lysates. However, immunoprecipitation using a monoclonal, monospecific antisera clearly revealed expression of both p10 and p17 (Fig. 3, lanes 4–9).

Qualitatively, the authentic and p17opt mRNAs produced limited amounts of p10 (Fig. 3, lanes 4 and 6), consistent with the inefficient natural translation start site of the p10 ORF in these constructs, and the p17 polypeptide was expressed at a moderate level from the authentic mRNA with the p17 start codon in a strong, though not optimal, sequence context (Fig. 3, lane 7). Optimizing the context of the p10 (Fig. 3, lanes 4 versus 5) or p17 (Fig. 3, lanes 7 versus 9) start sites resulted in an obvious increase in translation of these ORFs. Most notably, increased p10 expression from the p10opt mRNA led to the concurrent dramatic reduction of p17 translation (Fig. 3, lanes 7 versus 8). This observation was consistent with numerous previous studies indicating that ribosomes rarely scan past an optimized translation start site without initiating translation (1), providing the first direct evidence for coordinated expression of the p10 and p17 ORFs by leaky scanning initiation. In marked contrast, an increased number of scanning 40 S subunits initiating translation at either the optimized p10 or p17 start codons had little apparent inhibitory effects on αC translation (Fig. 3, lanes 10–12).

Translation of the αC ORF Is Independent of Leaky Scanning—Because in vitro translation systems may allow aberrant internal translation initiation (25, 26), rigorous quantitative analysis of the influence of the upstream ORFs on translation initiation at the internal αC start site was conducted in transfected cells expressing the various S1 mRNA constructs. The low avidity of the p17 antisera and/or limited expression of p17 in vivo (14) rendered quantitative analysis of p17 expression problematic; therefore, only p10 and αC expression were further examined.

Cell lysates from cells transfected with the pARV-S1, p10opt, and p17opt expression plasmids were fractionated by SDS-PAGE using dilutions of the lysates (i.e. sequential 1-μg decreases in the protein load) to obtain a linear detection range by Western blotting, and expression levels were quantified and normalized to actin controls using phosphorimaging (Fig. 4A). The relative mRNA levels were also quantified by qRT-PCR using the ΔΔC_T method (21). In no instance did variations in protein expression of the various S1 transcripts reflect significant differences in transcript levels (data not shown). Quantitative analysis indicated that p10 expression was increased an average of 6.1 ± 0.8-fold (n = 7) in p10opt-transfected cells relative to cells transfected with either the pARV-S1 or p17opt

![Image](https://example.com/image)
plasmids (Fig. 4B). This substantial increase in p10 expression was not reflected in a corresponding decrease in σC expression, which was reduced by 2-fold (i.e. σC expression from the p10opt mRNA was retained at $52 \pm 6\%$ of the levels obtained from pARV-S1). Optimizing the p17 start site had even less of an effect on σC expression, which was reduced by only $19 \pm 9\%$ compared with σC expression from the pARV-S1 construct, a difference that was not statistically significant (Fig. 4B).

The quantitative analyses of protein steady-state levels by Western blotting were confirmed by pulse-labeling and radioimmunoprecipitation. Extended exposure of the fluorograms detected low levels of p10 translation from the authentic p10 start codon in cells transfected with either the pARV-S1 or p17opt S1 expression plasmids (Fig. 4C). As with the Western blotting results, an optimized p17 start site had no statistically significant effect on σC translation, which was retained at $89 \pm 3\% (n = 5)$ that observed from the pARV-S1 mRNA. Similarly, optimizing the context of the p10 start site resulted in a dramatic increase in p10 translation with only a marginal decrease in σC translation (i.e. reduced by $22 \pm 4\%$ of the levels obtained from the authentic S1 mRNA construct) (Fig. 4C).

Therefore, results obtained both by optimizing the context of upstream start sites (Figs. 3 and 4) and by removal of these start codons (Fig. 2B) indicated that the mechanism responsible for σC translation provides ribosome access to the internal σC site in a reasonably efficient manner and relatively independent of the uORFs present in the full-length, tricistronic mRNA. We infer that few if any pre-initiation complexes access the σC translation start site via leaky scanning.

**Translation of σC Is IRES-independent—**The ARV and NBV S1 mRNAs were screened for the presence of an IRES element using the classical “bicistronic” approach (27). As a positive control for detection of IRES activity, a bicistronic vector was created in which the EGFP reporter gene under the control of the poliovirus IRES element was cloned downstream of the ARV S1 gene. A low level of EGFP expression suggestive of IRES-mediated internal translation initiation was detectible from this construct (Fig. 5A). Co-expression of the poliovirus 2A protease enhanced translation of the downstream EGFP ORF by 4-fold, consistent with the ability of the 2A protease to selectively induce cleavage of the eIF4G component of the eIF4F cap binding initiation complex, thereby decreasing cap-dependent translation while increasing cap-independent IRES
mediated translation initiation (28, 29). The ability of the 2A protease to inhibit cap-dependent translation was evident by the decreased expression of the 5′-proximal p10 ORF in the S1 cistron present in this bicistronic construct (Fig. 5A), and cleavage of eIF4G was confirmed by Western blotting (see Fig. 6C).

To screen for an IRES element in the S1 mRNA sequence, similar bicistronic vectors were created with the entire ARV or NBV S1 genes cloned downstream of the EGFP-encoding ORF (Fig. 5B). Radioimmunoprecipitation revealed robust expression of the 5′-proximal EGFP ORF present in these constructs, but there was no evidence of σC expression from the downstream S1 cistrons, even using the sensitive radioimmunoprecipitation procedure and extended exposure of the fluorograms (Fig. 5B). Furthermore, co-expression of the S1 mRNA with 2A protease to enhance IRES activity actually inhibited, rather than enhanced, σC expression when assessed by Western blotting (see Fig. 6B). There was, therefore, no evidence to indicate that σC translation initiation on the ARV or NBV S1 mRNAs is dependent on a sequence- or structure-based cis element that facilitates direct internal ribosome entry.

**Translation from the Internal σC Start Site Is eIF4G-dependent**—The accumulated evidence indicated σC translation initiation occurs from a full-length, tricistronic mRNA in a leaky scanning- and IRES-independent manner. The only other described mechanisms for translation initiation at internal AUG start codons are reinitiation and ribosomal shunting, both of which require the canonical cap-dependent initiation machinery (8, 30). To explore the role of the eIF4F cap binding complex in the mechanism of σC translation, we utilized the poliovirus 2A protease to disrupt cap-dependent translation initiation. In cells co-transfected with the pPV2A plasmid expressing the 2A protease and a pEGFP reporter plasmid, a dose-dependent reduction in EGFP expression was observed by both fluorescence microscopy (Fig. 6A, panels a and b) and immunoprecipitation (Fig. 6B). The activity of the 2A protease was confirmed by Western blot analysis using a polyclonal anti-eIF4G antiserum, which detected the anticipated cleavage of eIF4G (Fig. 6C). The ~50% cleavage efficiency of eIF4G paralleled the transient transfection efficiency of the QM5 cells (data not shown), suggesting efficient cleavage of eIF4G in the majority of cells likely to be co-transfected with the EGFP reporter plasmid. Similar results were obtained in cells co-transfected with the pPV2A and ARV-S1 expression plasmids. Immunostaining transfected cells expressing only the S1 mRNA with a polyclonal antiserum that recognizes σC detected antigen-positive, multinucleated syncytial foci (Fig. 6C, panels a, b, c, and d), indicative of both σC expression and expression of the p10 fusion-associated small transmembrane protein that is solely responsible for syncytium formation (15). Co-transfection with the 2A protease expression plasmid led to a loss of syncytium formation and a dramatic reduction in antigen-specific staining (Fig. 6A, panel d). The inhibition of syncytogenesis and the previous Western blotting results (Fig. 5A) clearly indicated the ability of the 2A protease to inhibit cap-dependent p10 expression. A similar 2A protease-dependent inhibition of σC expression was suggested by the loss of antigen-positive foci and was confirmed by immunoprecipitation that revealed a dose-dependent reduction in σC expression similar to that observed for EGFP expression in co-transfected cells (Fig. 6B).

Expression of the poliovirus 2A protease can lead to decreased transcription in cells, potentially contributing to an indirect inhibition of translation (31). S1 transcript levels were, therefore, assessed by RT-PCR to strengthen the conclusion that σC translation initiation is directly dependent on eIF4G. Semiquantitative RT-PCR revealed that under co-transfection conditions resulting in a >90% inhibition of σC expression, there was no detectible decrease in S1 transcript levels (Fig. 6D). These results were confirmed by qRT-PCR experiments (n = 3) that indicated no negative effect of the 2A protease on S1 mRNA levels (data not shown). Because the 2A protease is not known to induce cleavage of any other canonical translation...
initiation factors (e.g. elf4E, elf4A, elf4B, elf3) (29), these results suggested that the mechanism responsible for \( \alpha \)C translation is dependent on intact elf4G and, by inference, on a functional elf4F cap binding complex.

Expression of \( \alpha \)C Is Independent of Translation Reinitiation—The evidence that \( \alpha \)C translation is leaky scanning- and IRES-independent but elf4G-dependent was consistent with the previous suggestion that ribosomes access the \( \alpha \)C start site by first translating the p10 ORF in a cap-dependent manner followed by translation reinitiation at the downstream \( \alpha \)C start codon (1). To further test this hypothesis, the p10 ORF was progressively truncated from the 5’-end, and the effects of these deletions on \( \alpha \)C translation were analyzed (Fig. 7A). Deletion of the 5’-terminal 79 nucleotides of the S1 mRNA (pARV-S1d79), which removes the 5’-UTR and the first 55 nucleotides of the p10 ORF, including the 2 in-phase p10 AUG initiator codons at nucleotides 25 and 34, eliminated both p10 and \( \alpha \)C expression. The same situation applied to pARV-S1d147, which removed approximately half of the p10 ORF. Further truncation to nucleotide 303, which removed the p17 start codon at position 293 and the in-frame methionine codon in the p10 ORF at position 298 (i.e. the last of the four AUG codons that lie upstream of the \( \alpha \)C start site), restored \( \alpha \)C expression (Fig. 7A). The 1.7 ± 0.4-fold increase in \( \alpha \)C expression (\( n = 3 \)) from this pARV-S1d303 construct closely paralleled the ~2-fold increase in \( \alpha \)C translation observed with the pARV-S1mono construct (Fig. 2B), although in both instances the modest increase in \( \alpha \)C expression was not statistically significant.

The truncation results were consistent with a role for the p10 ORF to escort ribosomes past the strong p17 start site, resulting in \( \alpha \)C translation by reinitiation after translation of the p10 ORF. However, the data were also consistent with an alternate hypothesis that the truncations altered a cis element involved in a non-linear shunting mechanism for \( \alpha \)C translation. To distinguish between these two possibilities, a stop codon was introduced into pARV-S1 to truncate the p10 ORF at nucleotide 243 (p10stop243 construct), 50 nucleotides upstream of the p17 start codon. Western blot analysis of lysates prepared from cells transfected with this modified S1 construct detected expression of the truncated 73-residue p10 polypeptide (Fig. 7). Contrary to the reinitiation model, however, terminating the p10 ORF upstream of the p17 start codon had no deleterious effect on \( \alpha \)C expression that was actually slightly increased by 1.6 ± 0.06-fold (\( n = 3 \)). As with previous experiments, qRT-PCR revealed no significant difference in transcript levels of the authentic versus modified S1 mRNAs (data not shown). To ensure that the proximity of the inserted stop codon to the p17 start codon was not an issue, a second construct was tested that contained a stop codon inserted in the p10 ORF after nucleotide 84 (p10stop84 construct), 209 nucleotides upstream of the p17 start site. Quantitative Western blot analysis revealed that terminating p10 translation well upstream of the p17 start codon also had no inhibitory effect on \( \alpha \)C expression (Fig. 7). These results cannot be explained in the context of a reinitiation model of \( \alpha \)C translation, indicating that ribosomes access the \( \alpha \)C start site using some form of non-linear ribosome migration.

DISCUSSION

Previous results demonstrated that the S1 genome segment of ARV is functionally tricistronic, encoding three independent gene products from sequential, partially overlapping ORFs (13, 14). Based on the placement of the p10 and p17 start codons and the suboptimal context of the p10 start site, leaky scanning presented as a probable mechanism for p17 expression. This prediction was confirmed by in vitro translation analysis, which indicated that p10 and p17 expression derive by a context-dependent leaky scanning mechanism. A more important question related to how the translational machinery accesses the internal \( \alpha \)C start site. Translation reinitiation or an IRES were considered two likely mechanisms for \( \alpha \)C translation (1, 19). Present results, however, indicate that these common means of providing leaky scanning-independent ribosome access to internal translation initiation sites are not operative on the ARV S1 mRNA. Additional studies revealed that the mechanism responsible for \( \alpha \)C translation delivers the translation machinery to the \( \alpha \)C start codon present on a full-length, tricistronic mRNA in a reasonably efficient and elf4G-dependent manner and relatively independent of translation of the preceding ORFs. We, therefore, propose that \( \alpha \)C translation initiation derives from a novel form of non-linear ribosome migration.

Quantitative analysis indicated that an optimized p10 start site resulted in a modest, but reproducible, decline in \( \alpha \)C translation (Fig. 4), suggesting the possibility that \( \alpha \)C translation...
might derive in part via leaky scanning. However, the 2-fold decrease in σC translation was not proportional to the ~6–10-fold increase in p10 translation; the same optimized p10 start site had a much more pronounced inhibitory effect on p17 translation in vitro (Fig. 3), and an optimized p17 start site had no negative effect on σC translation in vitro or in vivo (Figs. 3 and 4). Furthermore, the natural p17 start codon served as an effective barrier to leaky scanning in the 5′-truncated pARV-S1d79 and -d147 constructs (Fig. 7A). The simplest interpretation of the available evidence is that ribosomes do not access the σC start site by linear scanning of the 5′-proximal 629 nucleotides preceding this start codon.

An ~10-fold increase in ribosomes initiating translation at the optimized p10 start site could conceivably have indirect effects on σC translation initiation, perhaps by occluding a cis element that includes sequences in the vicinity of the short 5′-UTR and the p10 start codon. The suggestion that cis elements essential for σC translation may reside in the 5′-proximal region of the S1 mRNA is supported by the observation that a 79-nucleotide 5′ truncation eliminated σC expression (Fig. 7A). If specific cis elements required for σC expression exist in the 5′-proximal region of the S1 mRNA, they are unlikely to be highly stable secondary structures. This conclusion is based on the observation that σC expression was nearly identical in the pARVS1mono and pARV-S1d303 constructs (Figs. 2A and 7A). Because deletion of the 5′-terminal 303 nucleotides did not increase scanning ribosome access to the σC start site, a highly stable secondary structure that impedes ribosome migration does not appear to be a requirement for σC expression, at least not in the 5′-proximal half of the σC leader sequence.

A common mechanism by which ribosomes bypass upstream sequences to access start codons that are not 5′-proximal is through the use of IRES elements (7). Analysis of bicistronic constructs, however, failed to provide any evidence that σC translation is mediated by an IRES element. With the caveat that the additional nucleotide sequences present in the bicistronic mRNA might alter the structure and function of an IRES element, we conclude that some mechanism other than internal ribosome entry must guide ribosome access to the σC start site. This conclusion is supported by the data indicating that σC expression was diminished rather than enhanced when cap-dependent translation was inhibited by cleavage of eIF4G (Fig. 6). Excluding the hepatitis A virus IRES, none of the other characterized IRES elements, including the type I and type II IRES elements of the picornaviruses and those present in the pestiviruses and dicistroviruses, require intact eIF4F for their function (3). In view of the essential role of eIF4G in formation of the eIF4F cap binding complex, the most straightforward interpretation of our results is that σC translation is mediated by a cap-dependent, IRES-independent mechanism, at least in the context of the S1 mRNA constructs expressed in transfected cells.

The absence of methionine codons in the intergenic region between the p10 and σC ORFs and the size of the intergenic region (~300 nucleotides, allowing sufficient time for scanning subunits to reacquire the eIF2-GTP-Met-tRNAi ternary complex) would allow ribosomes terminating translation at the p10 ORF to scan the intergenic region and reinitiate at the σC start site. These features led to the suggestion that the p10 ORF may serve a dual function, encoding the p10 membrane fusion protein and serving to shepherd ribosomes past the strong p17 start site to allow them access to the σC start site via translation reinitiation (1). A similar use of upstream ORFs to facilitate downstream reinitiation by guiding ribosomes past a preferred start site may be operative on several cellular mRNAs, such as those encoding isoforms of GlyRS or the transcription factor C/EBPβ (32, 33). However, two lines of evidence indicate that translation initiation of the σC ORF does not derive from a reinitiation event after translation termination of the p10 ORF. First, optimizing the translation start site of the uORF generally results in a proportional increase in translation reinitiation at the downstream start codon (32), as might be expected by the increased availability of 40 S subunits after translation termination of the uORF. Such is not the case for the S1 mRNA, where an ~10-fold increase in p10 translation from the p10opt mRNA results in a modest decrease, rather than an increase, in σC translation (Fig. 4). More importantly, insertion of stop codons that terminate translation of the p10 ORF upstream of the p17 start codon had no negative effect on σC expression (Fig. 7). The strong context of the p17 start codon, with the most highly conserved and functionally most important A residue at position −3 (2, 34), suggested that this start codon should at least partially inhibit 40 S subunits from scanning past the p17 start site to access the σC start codon. As previously mentioned, the loss of σC expression in the pARV-S1d79 and -d147 constructs provides evidence that the p17 start codon does in fact serve as an effective barrier to leaky scanning. The inability of this start codon to block ribosome access to the σC start site in the stop insertion constructs clearly indicates that the mechanism of σC expression does not involve translation reinitiation. This is particularly true for the p10stop84 construct that contains 209 nucleotides between the inserted p10 stop codon and the p17 start site, a distance sufficient to allow the majority of scanning 40 S subunits to reacquire initiation factors and become competent for reinitiation (35–37). We see no way to reconcile these results with a translation reinitiation mechanism for σC expression.

The only other described mechanism for translation initiation at internal start codons is ribosomal shunting (8). The clearest example of shunting occurs in the plant pararetroviruses and involves the discontinuous scanning of a complex leader sequence containing multiple short ORFs and an extended hairpin structure. In both cauliflower mosaic virus and RTBV, translation of the 5′-proximal short uORF and the presence of an adjacent stem-loop structure are absolute requirements for efficient shunting (38–41). Based on the evidence presented here, we propose that σC translation reflects an atypical ribosomal shunting mechanism. The initial step in this mechanism most likely involves recruitment of the 40 S subunit with associated canonical initiation factors to the 5′-UTR of the S1 mRNA, most likely in an eIF4G- and cap-dependent manner. In view of the fact that a 40 S subunit occupies ~30–35 nucleotides (42), the entire 5′-UTR of the authentic S1 mRNA and extending 5–10 nucleotides past the p10 start codon would be occluded by the bound pre-initiation complex. Because an optimized p10 start site has no adverse effect on σC expression, this mechanism most likely involves recruitment of the 40 S subunit with associated canonical initiation factors to the 5′-UTR of the S1 mRNA, most likely in an eIF4G- and cap-dependent manner. In view of the fact that a 40 S subunit occupies ~30–35 nucleotides (42), the entire 5′-UTR of the authentic S1 mRNA and extending 5–10 nucleotides past the p10 start codon would be occluded by the bound pre-initiation complex. Because an optimized p10 start site has no adverse effect on σC expression, this mechanism most likely involves recruitment of the 40 S subunit with associated canonical initiation factors to the 5′-UTR of the S1 mRNA, most likely in an eIF4G- and cap-dependent manner. 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expression, shunting of the pre-initiation complex to the vicinity of the ςC start codon, bypassing the p10 and p17 ORFs, would require little to no scanning and no prior translation of a short uORF. This is markedly different from the situation with pararetroviruses where such events are required to divert the 40S subunits from the scanning to the shunting route (39).

The limited scanning and reinitiation-independent model of S1 mRNA shunting shares certain features ascribed to two alternate models of ribosomal shunting. Shunting on the adenovirus late mRNAs involves the cap-dependent recruitment of 40S subunits followed by limited scanning of these subunits a short distance (~40–60 nucleotides) into the 5′-proximal unstructured region of the tripartite leader sequence (43). A similar situation occurs on the Sendai virus P/C mRNA, where limited scanning of the 5′-UTR upstream of the first start codon at nucleotide 81 precedes shunting of the pre-initiation complex to the vicinity of the downstream start sites (10).

There are several notable differences between the proposed adenovirus and Sendai virus shunting mechanisms and the features of the reovirus S1 mRNA shunting process described here. For example, both leaky scanning and shunting function at approximately equal levels to direct translation of the same polypeptides encoded by the family of late adenovirus mRNAs (43). Similarly, the production of the same set of four nested C-proteins from the Sendai virus P/C mRNA was originally proposed to derive from a combination of leaky scanning and shunting (10, 44), but recent analysis indicates that the majority of the truncated C-proteins derive from protein processing, not shunting (45). The situation is quite different in the case of the S1 mRNA, where two mutually exclusive mechanisms of translation initiation direct production of distinct proteins. The first mechanism utilizes context-dependent leaky scanning to express the p10 and p17 ORFs, whereas the second mechanism, involving non-linear transfer of the pre-initiation complex from the 5′-UTR to an internal start codon, appears to be solely responsible for expression of the ςC protein.

There are also likely to be important differences in the factors that contribute to the choice of which translation initiation mechanism is employed. Translation mediated by the adenovirus tripartite leader undergoes a time-dependent transition from the use of both leaky scanning and shunting to entirely shunt-dependent initiation late in infection (43). This transition parallels the inhibition of cap-dependent translation in virus-infected cells, and interactions of the adenovirus 100K protein with eIF4G are involved in both the inhibition of cap-dependent translation and the enhanced shunting on the late adenovirus mRNAs (46, 47). In contrast, the expression of p10 and ςC follow a similar time course in virus-infected cells (14), indicating that the two translation initiation mechanisms operate on the S1 mRNA are not temporally regulated. It is also unlikely that a functional equivalent of the adenovirus 100K protein contributes to the selection of whether a pre-initiation complex bound to the S1 mRNA undergoes context-dependent leaky scanning or shunting. The only candidates for such a viral translation transactivator are the three gene products of the S1 mRNA, since ςC expression occurs in vitro and in vivo in the absence of any other viral proteins. The ςC and p10 proteins are unlikely to provide such a function; ςC is structurally and functionally equivalent to the mammalian reovirus σ1 cell adhesion protein that possesses no such translation enhancement activity (18, 19), and p10 is an integral membrane protein that is trafficked to the plasma membrane where it functions to induce cell-cell fusion and syncytium formation (15). Our current demonstration that optimizing the p10 start site dramatically reduces expression of p17 with minimal effects on ςC translation suggests p17 has little if any role to play in influencing the choice of translation initiation mechanism. We assume that cis elements present in the S1 mRNA influence the selection process that leads to leaky scanning or shunting, but aside from the extended hairpin structure that promotes pararetrovirus shunting (9, 38), cis elements that function as shunt donors or acceptors have not been well defined in the other examples of shunting. A group of stable hairpin structures that possess complementarity to the 3′-end of 18 S RNA were identified in the adenovirus tripartite leader (11, 48), but the role of these elements in the shunting process remains unclear. Random PCR mutagenesis of the 600-nucleotide leader sequence that precedes the ςC start site is currently under way to identify important cis regulatory elements in the S1 mRNA, which should provide important additional insights into what appears to be a novel mechanism of ribosome shunting regulating translation initiation on this remarkable tricistronic mRNA.

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Avian Reovirus Tricistronic S1 mRNA

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