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Translational Modulation In Vitro of a Eukaryotic Viral mRNA Encoding Overlapping Genes: Ribosome Scanning and Potential Roles of Conformational Changes in the P/C mRNA of Sendai Virus

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Expression of proteins from three overlapping genes in a single mRNA species of Sendai virus was modulated in a cell-free rabbit reticulocyte translation system. Hybrid-arrested translation by oligodeoxynucleotides complementary to specific regions of the mRNA that specifies the viral P, C, and C' proteins demonstrated that ribosomes scan the RNA from its 5' end to find initiation codons, and suggested that the secondary structure of the mRNA influences the selection of alternative initiation codons. Translational modulation of P, C, and C' proteins by Mg^{++} and spermidine indicated that RNA folding is involved in this selection process.

The genome of Sendai virus, a murine paramyxovirus closely related to human parainfluenza viruses, is a single-stranded RNA molecule of negative polarity (1). The virion contains a virus-encoded transcriptional apparatus which is responsible for the generation of monocistronic mRNA species specifying five proteins, NP, M, F_0, and L, and a sixth polycistronic mRNA that specifies three proteins, P, C, and C' (2,5). The P protein is found in the virus particle, but C and C' have not been observed in virions and have therefore been designated nonstructural. P is putatively involved in viral RNA-dependent RNA synthesis, but the functions of C and C' are unknown. Nucleotide sequence analyses of the P/C gene reveal a large open reading frame for the P protein and, within it, in a +1 reading frame, nested sequences encoding the two nonstructural proteins (2,5). Because the reading frames for C and C' are in the same phase, these proteins are structurally related (14). All available evidence indicates that a single species of mRNA is the template for the three proteins (3-6). None of the AUG initiation codons in P/C mRNA

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have the most favorable context of neighboring nucleotides observed in most mRNAs of eukaryotes and of the viruses that infect them (7), so there is scope for modulation of translation at the level of initiation efficiency, along with the translational modulation possibilities afforded by the presence of overlapping genes.

Despite considerable evidence that eukaryotic ribosomes scan mRNAs for initiation codons in a linear fashion, starting at the 5' termini of the RNAs (7), evidence has been presented that calls this mechanism into question, suggesting that internal initiation is possible within certain polycistronic viral mRNAs (8). To address this issue, we applied a novel technique of arresting translation by hybridization with synthetic oligonucleotides designed to be complementary to selected regions of P/C mRNA (9,20). Our results demonstrate that ribosomes do indeed scan this mRNA from its 5' end and suggest further that conformation of the mRNA plays a role in the selection of initiation codons.

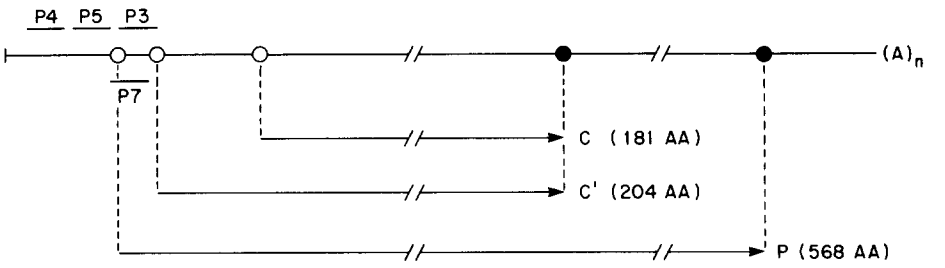
MATERIALS AND METHODS

mRNA and translation - Total cytoplasmic RNA from Sendai virus-infected chick embryo cells was used for *in vitro* translations (10). Reactions were run under the conditions suggested by the supplier of the rabbit reticulocyte lysate (BRL) in the presence of 5 to 10 µg of RNA and 15 to 30 µCi of [^{35}S]methionine (1300 Ci/mmol). Whenever oligonucleotides were added before translation, the RNA and oligonucleotide (0.1 µg of the latter per 20 µl translation reaction) were annealed by heating at 100°C for 3 minutes and then quickly cooled to 0°C.

Oligonucleotides - Oligodeoxynucleotides (18- to 21-mers) complementary to specific 5'-proximal regions of P/C mRNA were synthesized with an Applied Biosystems oligonucleotide synthesizer. The base positions and locations of the oligonucleotides relative to P/C mRNA are given in Fig. 1.

RESULTS

Hybrid-arrested translation - To determine if P/C mRNA was scanned by ribosomes starting at its 5' end, we reasoned that an oligonucleotide that hybridizes to a region within the 5' non-coding region should arrest the translation of all three proteins. Indeed, oligonucleotides P4 and P5, both of which hybridized to this region of the mRNA (Fig. 1), inhibited the translation of all three proteins (Fig. 2). The level of inhibition was slightly



Initiation codons	sequence context
P	CGCAUGG
C'	AAGAUGC
C	CGGAUGC
consensus of eukaryotic and their viral mRNAs	$^{A}_{G}$NNAUGPu

Figure 1. Coding frames of P/C mRNA and sequence contexts of initiation codons. Open circles represent the initiation codons for proteins P, C', and C at bases 104, 114, and 201, respectively, in the gene (2,5). Closed circles represent termination codons. Small horizontal bars give the positions of the complementary oligonucleotides used in the hybrid arrest experiments. Two of the oligonucleotides, P4 and P5, correspond to bases 41 through 60 and 71 through 90, respectively, in the non-coding region of P/C. P7 is complementary to bases 91 through 110 and spans the first initiation codon. P3 is complementary to bases 104 through 123, spanning the first two initiation codons. AA, number of amino acids in each protein.

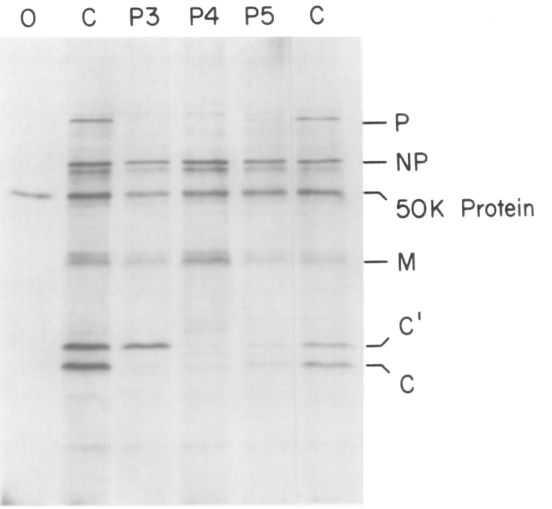


Figure 2. Polyacrylamide gel electrophoresis of translation products synthesized after hybridization of mRNA with specific oligonucleotides. Products were mixed with an equal volume of 2X Laemmli (15) sample buffer, heated at 70°C for 15 min and analyzed in a 10% polyacrylamide Laemmli (15) gel. C, translation products of RNA alone. P3, P4, and P5, products made after hybridization with the correponding oligonucleotides. O, products made in the absence of any added RNA.

less for P5 than for P4, perhaps reflecting a difference in hybridization efficiency. Oligonucleotide P7, which spans the first initiation codon, where translation of the P protein begins, similarly inhibited translation of all three proteins (data not shown). We conclude that ribosomes must scan this mRNA from its 5' end to initiate translation at any site.

However, when oligonucleotide P3, which spans the first two initiation codons, was used to arrest translation, an unexpected result was obtained. P3 inhibited translation of both P and C proteins, but not C' (Fig. 2), suggesting that something other than the primary structure of mRNA, such as secondary or higher order structure (18), influences the selection of initiation codons.

Influence of mRNA conformation on its translation - To test whether conformation of P/C mRNA has a role in the selection of initiation codons, we used inducers and stabilizers of secondary and higher order structures in nucleic acids, such as Mg^{++} and spermidine (12,13,19). In the absence of extra Mg^{++} (the concentration of endogenous Mg^{++} required for efficient cell-free translation was about 1 mM), there was a decrease in C' synthesis, but no effect on the production of P or C (Fig. 3, lane 5). When the total concentration of Mg^{++} was increased to 2 mM, the level of C' synthesis increased without an effect on P or C synthesis (Fig. 3, lane 1). A higher concentration of Mg^{++} (3 mM) totally blocked translation (data not shown).

Spermidine at 0.5 mM substituted for Mg^{++} in supporting a full level of C' expression (Fig. 3, lane 7). However, with 2 mM Mg^{++} and 0.5 mM spermidine, synthesis of C was markedly inhibited and some suppression of P synthesis was seen (Fig. 3, lane 3). Spermidine at 0.05 mM (with or without extra Mg^{++}), had no effect (Fig. 3, lanes 2 and 6), whereas at 2.5 mM (again irrespective of added Mg^{++}), spermidine blocked the whole translation process, except for the synthesis of a non-viral Mr 50,000 protein (Fig. 3, lanes 4 and 8). Clearly, Mg^{++} and spermidine independently as well as synergistically affect the expression of all three proteins encoded in P/C mRNA.

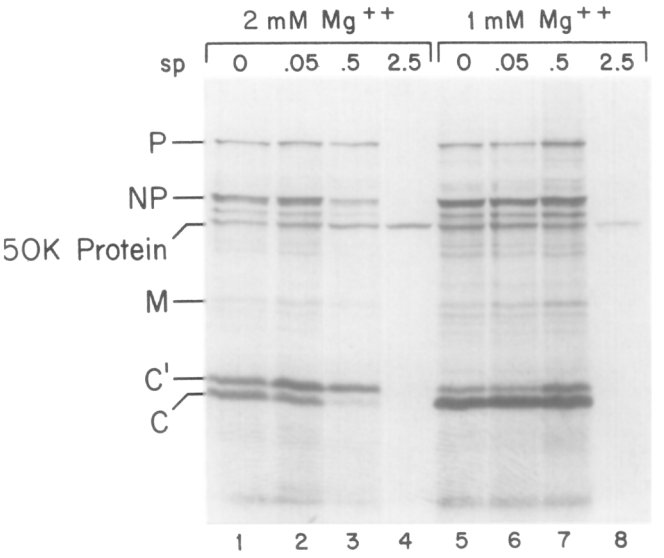


Figure 3. Effects of Mg^{++} and spermidine on the synthesis of P, C, and C' proteins. Reactions shown in lanes 1 through 4 were run in 2 mM Mg^{++} and reactions in lanes 5 through 8 were run in 1 mM Mg^{++}. Supplementary Mg^{++} was added as Mg acetate. sp, levels of spermidine (mM), given above each lane. Electrophoresis was in a 10% polyacrylamde gel (16) containing 7M urea.

DISCUSSION

Translational inhibition of all three proteins encoded in P/C mRNA by oligonucleotides P4, P5, and P7, hybridizing either to the 5' non-coding region or spanning the first initiation codon, shows that the mRNA must be scanned from its 5' terminus for recognition of all of its initiation codons. In addition, the modulation of P, C, and C' synthesis by oligonucleotide P3, Mg^{++}, and sperimidine suggests that RNA folding plays a role in the selection of initiation codons, as originally proposed by Kozak (11). Recently, Rietveld et al. (19) observed that the three-dimensional folds of three plant virus RNAs were reduced by lowering the Mg^{++} concentration. Thus, changes in secondary or higher order structure around or at an initiation codon may affect the amounts of individual proteins synthesized from an mRNA like P/C, containing overlapping genes.

A search for hairpin structures (16) in the mRNA revealed three possibilities in the 5' non-coding region and at the initiation codons of P, C, and C'

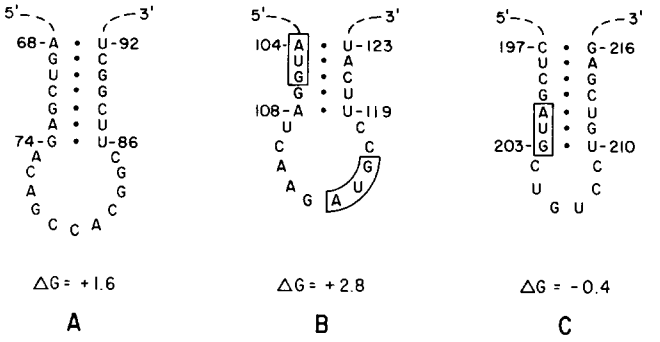


Figure 4. Hypothetical hairpins in P/C mRNA. Complementary matches were found by Staden's (16) HAIRGU program. Only relevant regions of the RNA species are shown. Free energies (ΔG) of the hairpins were estimated according to Tinoco et al. (17).

(Fig. 4). Based on free energy calculations (17), the stabilities of these putative hairpins (labeled A, B, and C in the figure) take the order C:ΔG=-0.4, A:ΔG=1.6, and B:ΔG=2.8. Hairpin B, the least stable, may modulate the expression of proteins P and C', because it embraces their initiation codons. It has a 5 base-pair stem including one U-G pair and a 10-base loop, The initiation codon of P resides in the stem and that of C' lies in the loop. Mg^{++} and spermidine would stabilize such a structure (13), perhaps causing the ribosome to pause and initiate protein synthesis in this region. Initiation of C' might be favored somewhat over P (Fig. 3, lane 3) because the C' initiation codon is exposed in the loop and the sequence context of this codon is more favorable, due to the presence of an A residue 3 bases upstream (Fig. 1).

Markedly preferential synthesis of C' in the presence of oligonucleotide P3 suggests that a linear duplex, by eliminating the possibility of hairpin formation, directs the ribosome to the initiation codon that possesses the strongest context. Presumably, hairpin A is too far upstream to exert any modulating effect, and hairpin C could come into play only when initiation at one of the upstream sites failed to occur. A search for long-range base pairing throughout the whole P/C mRNA (16) did not reveal structures at or around the initiation codons or elsewhere that were more stable than those shown in Fig. 4.

In cells infected by Sendai virus, P protein is preferentially synthesized over C and C', whereas enhanced synthesis of the nonstructural proteins is observed in cell-free extracts, for unknown reasons (6,14). Therefore, the relevance of the present *in vitro* studies to the regulation of Sendai virus protein synthesis *in vivo* is not clear. But, since, *in vivo*, P protein is made more abundantly than C, whereas C' is barely detectable (6,14, and our unpublished results), it seems likely that formation of the hypothetical hairpin B is very rare *in vivo*. Intermittent failure of initiation of P (or C') would then allow initiation of C downstream. These speculations will require further experimental testing, and it remains to be seen whether RNA folding plays a generalized role in the selection of initiation codons of mRNAs that contain overlapping genes.

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