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Translation of bicistronic viral mRNA in transfected cells: Regulation at the level of elongation

(reovirus/scanning/initiation/bovine papilloma virus)

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ABSTRACT **The S1 species of mammalian reovirus mRNA, like a number of other viral but not cellular mRNAs, codes for two dissimilar polypeptides by initiation of translation at two 5′-proximal, out-of-frame AUG codons. To determine if uninfected cells can utilize bicistronic genes, a bovine papilloma virus-based vector system was used to select mouse C127 cell lines containing multiple integrated copies of the reovirus *S1* gene. These cell lines produced both reovirus polypeptides from a single mRNA. In addition, studies of COS cells transfected with the *S1* gene containing small changes around the first AUG suggest that bicistronic mRNA translation is regulated at the level of elongation. A model is proposed in which ribosomes engaged in translation of one reading frame interfere with movement of ribosomes in the other frame because of differences in codon usage. Expression of bicistronic genes may be similarly regulated in virus-infected cells.**

Eukaryotic mRNAs are generally monocistronic, and selection of the translational initiation codon is strongly position dependent—in most cases restricted to the first AUG codon (1). According to the scanning model for initiation, mRNAs are accessed at the capped 5′ end in a process that is mediated by cap-binding protein(s) (1, 2). The 40S ribosomal subunit with associated factors and ternary complex starts to scan along the message from the 5′ entry site (3). When the first start codon is encountered, a 60S subunit usually but not always joins to form the 80S ribosome, and the cistron is translated.

In a modified version of the scanning model, nucleotides in the immediate vicinity of an initiation codon play an important role in determining the efficiency of its utilization (4). Thus, the sequence GCCGCC (G or A) CCAUGG has emerged as a consensus for "strong" initiation codons in animal cell and viral messages, with the purine (usually A) at the −3 position and the G at +4 being most influential (the A of AUG is numbered +1, and nucleotides to the left are negative). Ribosomal subunits reaching an AUG that lies in a "weak" context—i.e., departing from the consensus (especially the −3 and +4 positions)—may either initiate protein synthesis at that site or continue the migration in search of a downstream initiation codon. Other mRNA features such as stable secondary structures involving the 5′ untranslated leader (−50 kcal/mol; ref. 5) and close proximity of the first AUG to the cap (1, 6) have also been correlated with reduced efficiency of initiation.

Although examples of cellular mRNAs that initiate at more than one AUG have been described, truly bicistronic mRNAs—i.e., those encoding two completely dissimilar peptides (rather than N-terminally extended identical sequences)—are limited to viral genes (7). The mammalian reovirus *S1* gene is transcribed into a bicistronic mRNA that encodes two different proteins in overlapping reading frames (8, 9). In the serotype 3 S1 message, the first open reading frame, which codes for the 49,126-Da σ1 viral hemagglutinin, starts at nucleotide 13 from the capped 5′ end and spans the 1.4-kilobase (kb) mRNA almost in its entirety before terminating 36 nucleotides from the nonpolyadenylylated 3′ end. The second open reading frame starts at nucleotide 71 and encodes p14, a nonstructural polypeptide of M_r 14,017. Synthesis of p14 from the second AUG in the reovirus S1 message may be facilitated by the suboptimal sequence context (C in the −3 position) and the short distance (13 nucleotides) from the 5′ terminus of the σ1 AUG.

To explore these possibilities, constructs were made with a cDNA copy of the S1 gene coding region containing different sequences around the first initiator AUG. These plasmids were used to express the *S1* gene products both stably and transiently in mammalian cells. The results demonstrate that transfected cells are capable of utilizing both the σ1 and p14 open reading frames. Evidence is also provided for regulation of protein synthesis at the level of elongation, resulting from simultaneous translation of two different reading frames on the same mRNA coding region.

MATERIALS AND METHODS

Enzymes and Reagents. Restriction endonucleases were obtained from BRL and New England Biolabs. Calf intestine alkaline phosphatase, T4 polynucleotide kinase, and S1 nuclease were purchased from Boehringer Mannheim; and Moloney murine leukemia virus reverse transcriptase and T4 DNA ligase were from BRL. The nick-translation kit and T4 DNA polymerase were from IBI. Assay conditions were according to suppliers' recommendations. All tissue culture supplies were obtained from GIBCO. Radioactive nucleotides and amino acids were purchased from Amersham, and ^{14}C-labeled molecular mass markers for protein gels were from Amersham and BRL.

Construction of Expression Vectors. Plasmids pBC12BI (10) and pB12-5 (11) were used to construct expression vectors containing the indicated modifications at the σ1 start site (Fig. 1). All of the constructs were verified by dideoxynucleotide DNA sequencing of alkali-denatured supercoiled plasmids (14).

A bovine papilloma virus (BPV)-based vector was used for the selection of stable cell lines expressing the products specified by the reovirus *S1* gene. Plasmid pBMTX contains the entire BPV-1 genome fused to a portion of pBR322 and a 3.8-kb fragment containing the mouse metallothionein I gene (15). pBMTX DNA was linearized at the unique *Xho* I site downstream of the metallothionein promoter; after filling the ends by using reverse transcriptase, the DNA was ligated to the blunt-ended *Hin*dIII–*Xmn* I fragment isolated from pCOS-S1. Colonies were analyzed by restriction enzyme digestion of extracted DNA.

Cells. Mouse C127 cells and monkey kidney COS 7 cells, obtained from B. Cullen, were grown in Dulbecco's modified

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Abbreviations: BPV, bovine papilloma virus; SV40, simian virus 40.
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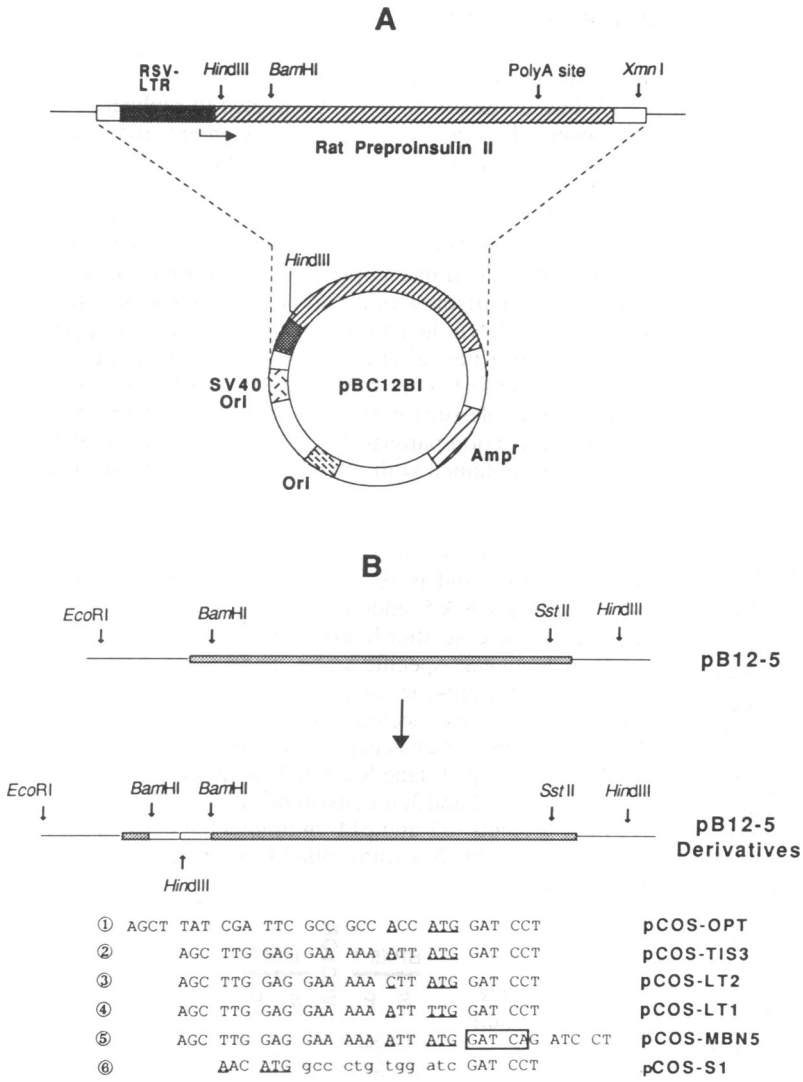

FIG. 1. Vectors for transient expression. (*A*) Plasmid pBC12BI (10), which includes an origin of replication (Ori) and other sequences (clear regions) from pBR322, an ampicillin-resistance gene (Amp^r), the simian virus 40 (SV40) origin that allows large tumor antigen-dependent replication in COS cells (SV40 Ori), and a Rous sarcoma virus long terminal repeat (RSV-LTR) was modified for expression of the two S1 polypeptides in COS 7 cells. Also indicated are the rat preproinsulin II gene with its poly(A) addition site and restriction enzyme sites used for cloning. (*B*) Plasmid pB12-5 (11) contains a complete cDNA copy of the *S1* gene (shaded box) in the polylinker region of pEMBL9 (+) (ref. 31). Sequences adjacent to the first ATG of the *S1* gene were modified by ligation of a 20-fold molar excess of phosphorylated oligonucleotides carrying *Bam*HI and *Hin*dIII staggered ends (open boxes) with *Bam*HI-linearized pB12-5. To construct pCOS vectors 1–4, the appropriate *Hin*dIII fragment from the corresponding pB12-5 derivative was inserted at the unique *Hin*dIII site of pBC12BI, resulting in plasmids containing the indicated sequences at the 5′ end of the *S1* gene. pCOS vector 5, obtained by *Bam*HI restriction and reverse transcriptase filling of a pCOS-TIS3-related plasmid lacking the insulin *Bam*HI site, contains a four-nucleotide duplication and a fortuitous extra A residue (boxed sequence), which results in a frame shift and termination of the σ1 reading frame after 10 amino acids. The transcripts from these constructs start at the RSV-LTR cap site and include 34 nucleotides of LTR sequence (nucleotides 230R–263R in ref. 12) followed by an extra C before the *Hin*dIII site. For construction of pCOS vector 6, the *Bam*HI–*Sst* II fragment of the *S1* gene was blunt-ended with T4 DNA polymerase and ligated to *Bam*HI-linearized, blunt-ended pBC12BI. Translation of transcripts from this construct initiates at the preproinsulin strong AUG initiator (13) and reads four codons (small letters) before the σ1 sequence. The initiator triplet and nucleotide at the −3 position are underlined.

Eagle's medium (DMEM; high glucose) supplemented with 10% fetal bovine serum.

DNA Transfections. For construction of stable cell lines, DNA coprecipitated with calcium phosphate was used to transfect C127 cells (11, 16). For transient expression assays (10), COS 7 cells were seeded at 4×10^5 per 60-mm plate in DMEM supplemented with 10% fetal bovine serum. Twenty hours later the medium was removed, and the monolayers were washed twice with phosphate-buffered saline (PBS). DEAE-dextran (Pharmacia), dissolved in PBS at 1 mg/ml, was mixed with 2 μg of plasmid DNA and applied to each plate (0.5 ml) followed by incubation for 30 min at 37°C, shaking occasionally to prevent drying. Next, 6 ml of supplemented medium containing 60 μM chloroquine (Sigma) was overlaid, and the cells were incubated at 37°C for another 4 hr. Finally, the medium was removed, and the cells were washed twice with PBS and kept at 37°C in 8 ml of supplemented medium. After 40–50 hr, the cellular proteins were radiolabeled and analyzed.

Analysis of Transformants. To label cellular proteins of transfected cells, the medium was removed, and, after the monolayer was washed twice with 2 ml of PBS, 1 ml of methionine-free medium containing 150 μCi (1 μCi = 37 kBq) of [^{35}S]methionine (approximate specific activity, 1000 Ci/mmol) was added to each plate. After 4 hr at 37°C, cells were harvested and lysed in 0.6 ml of buffer I [1% Triton X-100/1% sodium deoxycholate/0.35 M NaCl/10 mM EDTA/1% sodium dodecyl sulfate (SDS)/25 mM Tris·HCl, pH 7.5]. Aliquots of radioactive cell lysates were either precipitated with 10% cold trichloroacetic acid or analyzed by immunoprecipitation followed by SDS/polyacrylamide gel electrophoresis (17) and fluorography (Autofluor, National Diagnostics, Manville, NJ). For analysis of stable cell lines, transcription from the metallothionein promoter was induced by $CdCl_2$ addition 4 hr prior to radiolabeling.

Analysis of Cellular RNA and DNA. DNA isolated as described (18) and separated into high and low molecular weight fractions (19) was analyzed by agarose gel electrophoresis. The gel was treated for 20–30 min with 1 M KOH to denature the DNA and neutralized for 1 hr by addition of 75 ml of 1 M Tris base/1 M HCl per 100 ml of alkali. Finally, the gel was soaked for 15–30 min in 6× SSC (1× SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), and DNA was transferred to nitrocellulose (presoaked in 2× SSC) overnight with 10× SSC as the transfer buffer. The nitrocellulose was then processed by standard procedures (20). RNA was isolated as described (21).

Nick-translation was done on the gel-purified *Bam*HI–*Sst* II fragment of the *S1* gene, and unincorporated nucleotides were removed from the reaction mixture by spin filtration through Sephadex G-50 medium DNA grade (Pharmacia). The specific activity of the DNA probes was typically 2×10^8 cpm/μg.

S1 nuclease analysis was done with 40 μg of total cytoplasmic RNA as described (22). For preparation of the probe, pCOS-S1 DNA was digested with *Mlu* I, which cuts the

plasmid once at a site corresponding to position 370 of the *S1* gene—i.e., 299 nucleotides downstream of the p14 initiator codon. Restriction enzyme digestion was followed by treatment of the DNA with calf intestine alkaline phosphatase. After inactivation of the phosphatase, dephosphorylated DNA was labeled at the 5′ end by using [γ-^{32}P]ATP and T4 polynucleotide kinase and then was digested with *Hin*dIII, which cuts the vector upstream of the *S1* gene. The ≈540-bp labeled fragment was isolated from a 1.1% agarose gel by freezing the agarose slices in 20 mM Tris, pH 7.5/0.2 M NaCl/20 mM EDTA (Elutip low-salt buffer) followed by purification through an Elutip column (Schleicher & Schuell) and was used as a probe for the S1 nuclease analysis.

RESULTS

Mouse C127 cells were transfected with plasmid BPX5, which contains the complete cDNA coding sequence of the reovirus serotype 3 *S1* gene downstream of the mouse metallothionein I promoter (11, 15). Foci of transformed cells were grown and analyzed by immunoprecipitation of [^{35}S]methionine-labeled cell lysates with specific antibodies to the primary translation products of the *S1* gene. Fig. 2*A* shows the results with anti-σ1 antibody F7 (23) and lysates prepared after induction of one of the transformants (BPX5-4) at increasing $CdCl_2$ concentrations. An immunoprecipitated protein that migrated slightly above the 46-kDa molecular mass marker, the position expected for the σ1 polypeptide, increased in response to $CdCl_2$ to a maximal level at 1 μM. Analysis of lysates with rabbit antiserum 127 prepared against a synthetic peptide corresponding to amino acids 30–42 of p14 (24) detected low levels of a protein of the expected size in the presence (lane +) but not in the absence (lane −) of $CdCl_2$ (Fig. 2*B*). When these same antibodies were used to analyze cell lysates from $CdCl_2$-treated BPV-transformed C127 cells that do not contain *S1* gene sequences, no 49-kDa or 14-kDa bands were present (control in Fig. 2*B*), demonstrating the specificity of the immunoprecipitation. (The band at ≈60 kDa is an unidentified cellular protein that cross-reacts with antiserum 127.) Production of both σ1 and p14 in the same cell line suggests that, as in reovirus-infected mouse cells, the *S1* gene-derived message is functionally bicistronic in uninfected cells.

BPV-based vectors have been reported to replicate extrachromosomally as multicopy episomal elements in the nuclei of transformed cells (16). To determine the state of the DNA in BPV transformant BPX5-4, total cellular DNA was analyzed by Southern blot hybridization to a nick-translated *S1*-specific probe (Fig. 3). The results show that the DNA was present in multiple copies per transformed cell, estimated by comparison with the hybridization signal obtained with plasmid DNA in amounts corresponding to approximately one copy per cell (Fig. 3, lane vector). In addition and in contrast to most other BPV vectors, the DNA was integrated at multiple sites in the cell genome, as shown by the complex restriction pattern (Fig. 3, BPX5-4 lane P). Similar results were obtained with other independent transformants (data not shown).

To ensure that cell line BPX5-4 does not make a truncated S1 mRNA species that has its 5′ end between the σ1 and p14 initiator codons and is responsible for p14 production, we analyzed the mRNA 5′ end(s) by digestion with single strand-specific S1 nuclease after hybridization with a 5′-end-labeled ≈540 nucleotide *S1*-specific probe. The results of the analysis demonstrate the presence of a single, 387-nucleotide protected fragment—the size expected of a fragment extending from the *Mlu* I site to the splice acceptor site upstream of the preproinsulin AUG [Fig. 4, lane S1(+)]. This finding, together with the data in Figs. 2 and 3, is consistent with synthesis of the two reovirus proteins (σ1 and p14) in uninfected cells by translation of a single mRNA transcribed from integrated copies of the *S1* gene.

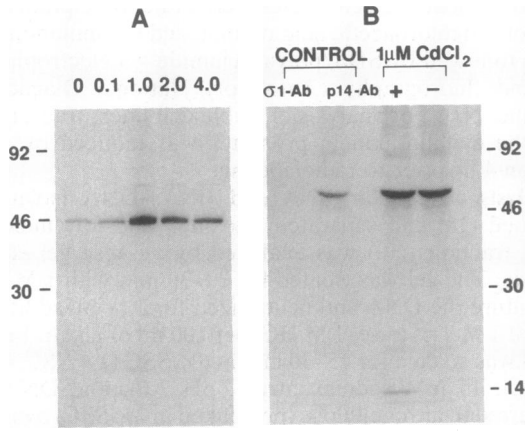

FIG. 2. Expression of viral proteins in cells stably transfected with the *S1* gene. Mouse C127 cells transformed with plasmid BPX5 were induced with $CdCl_2$ at the indicated concentrations, and [^{35}S]methionine-labeled lysates were immunoprecipitated. (*A*) Immunoprecipitation with σ1-specific monoclonal antibody F7 (23). The position of molecular mass markers in kDa is shown on the left. The faint bands above σ1 in lanes 1.0, 2.0, and 4.0 are of unknown origin. (*B*) Immunoprecipitation with antibody 127 (24). A $CdCl_2$-inducible protein migrating at the position expected for p14 is evident in BPX5-transformed C127 cells (lane +) but not in $CdCl_2$-treated control cells containing the vector alone (lane p14-Ab). The first lane shows the immunoprecipitate of control cell lysate with antibody F7 and demonstrates the specificity of the immunoprecipitation in *A*.

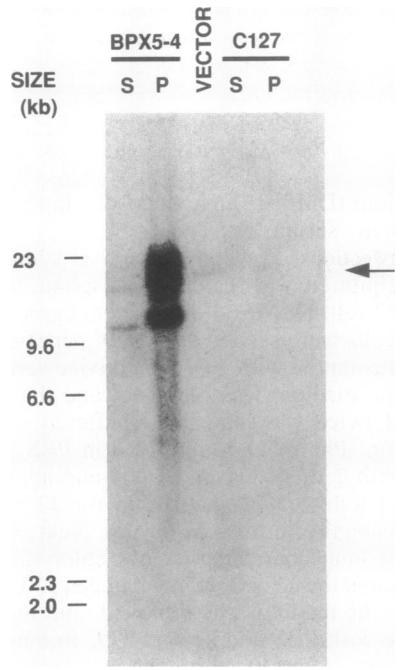

FIG. 3. Integration of multiple copies of the *S1* gene in transfected C127 cells. DNA prepared from C127 cells and *S1* gene-transformed cell line BPX5-4 was separated into high (lanes P) and low (lanes S) molecular weight fractions (19). The DNA was digested with *Bam*HI, which cuts the plasmid once outside the *S1* gene, and was subjected to electrophoresis in a 0.7% agarose gel. After transfer to nitrocellulose, the DNA was hybridized to a nick-translated *S1*-specific probe, and the membrane was used for direct autoradiography. The lane labeled "vector" was loaded with 90 pg of *Bam*HI-linearized BPX5 DNA (indicated by the arrow). The position of size markers is shown at the left.

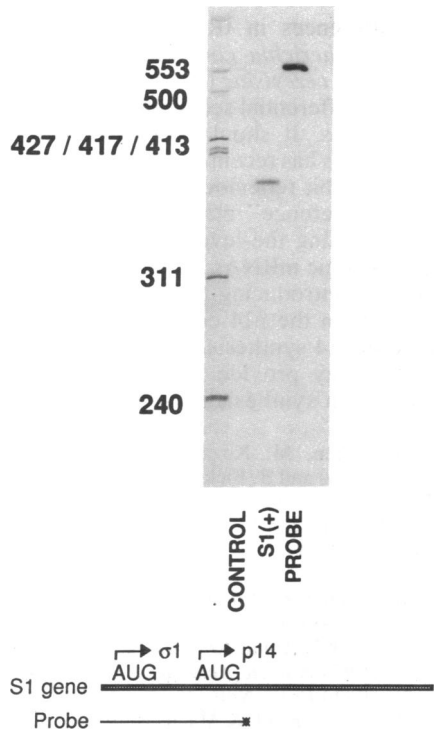

FIG. 4. (*Upper*) Analysis of the 5′ end of S1 transcripts. Total cytoplasmic RNA isolated from C127 cells (control) and from BPX5-4 cells [lane S1(+)] was hybridized to a 5′-end-labeled, ≈540-nucleotide probe specific for the *S1* gene and digested with single strand-specific S1 nuclease. The digested samples were analyzed in a 6.5% polyacrylamide/8 M urea gel and visualized by autoradiography. Sizes of the *Hin*fI-digested φX174 replicative form DNA markers are indicated in nucleotides. (*Lower*) The position of the label in the probe relative to the σ1 and the p14 initiator AUGs is shown schematically.

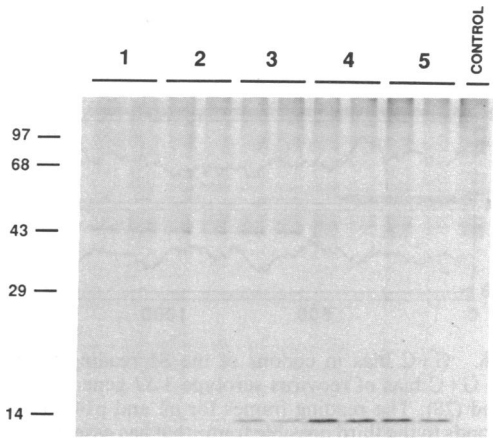

FIG. 5. Transient expression of S1 polypeptides. COS-7 cell monolayers were transfected in duplicate with plasmids carrying small sequence modifications around the σ1 AUG initiator codon. The relative levels of σ1 and p14 produced were analyzed by densitometry of autoradiographs after immunoprecipitation of [^{35}S]-methionine-labeled lysates with antibody made against gel-purified p14, which also cross-reacts with σ1 (11). Lanes 1–5 contain duplicate immunoprecipitates of lysates from cells transfected with the constructs numbered correspondingly at the bottom of Fig. 1. Control lysates are from cells transfected with pBC12BI, the original vector with no insert. Polypeptide σ1 can be seen in lanes 1–3 slightly above the 43-kDa marker. Polypeptide p14, which migrates at the position of the 14-kDa marker, is present in all samples (except the control), including cells transfected with plasmid pCOS-OPT, where the σ1 AUG is in a strong consensus sequence (seen in the original but too faint to be reproduced in photograph).

In reovirus-infected mouse cells, both proximity to the 5′ end and suboptimal sequence context of the first AUG in the S1 message may contribute to the observed bicistronic translation. The results presented here demonstrate that in BPX5-4, an uninfected mouse cell line, translation of p14 is initiated at the same internal AUG as in the case of the authentic viral message made in virus-infected cells (8). This occurs in the stably transfected cells despite a 5′ untranslated leader lengthened to >100 nucleotides and a strong sequence context around the first AUG in the engineered, chimeric S1 message.

Bicistronic mRNA translation was also studied in a transient expression system using COS 7 cells and SV40-based vectors. Several sequence variations were made around the first (σ1) AUG in the *S1* gene to determine how small sequence changes may affect the translation of each reading frame in the bicistronic message, as measured by the relative levels of σ1 and p14 in transfected cells. As in stably expressing cells, S1 nuclease analyses of mRNAs from transfected COS cells indicated a single 5′ end with each of the constructs, consistent with bicistronic function (data not shown). p14 levels decreased as the sequences surrounding the upstream σ1 initiation codon approached the consensus sequence (Fig. 5). Inactivation of the first (σ1) AUG by mutation to UUG resulted in an increase in p14 production by a factor of 3.5 (Fig. 5, lanes 4 relative to 2). A similar increase (factor of 3.0) in p14 synthesis was also attained by insertion of a termination codon 10 triplets downstream of the initiation site for σ1 synthesis (Fig. 5, lanes 5 relative to 2). This result is consistent with efficient reinitiation of translation (25, 26) by ribosomes engaged in translation of the short, prematurely terminated σ1 peptide. In contrast to the results obtained for p14 synthesis, which are predicted by the modified scanning model, the levels of σ1 apparently did not change as the sequences surrounding the initiator codon were altered (compare lanes 1 and 3). Similar results (including quantitative precipitation) were obtained with anti-σ1 antibody F7 (data not shown). Possible explanations of these findings are discussed below in the context of the modulation of p14 levels in reovirus-infected cells.

DISCUSSION

The reovirus *S1* gene is functionally bicistronic in virus-infected cells and, as demonstrated in this report, in transfected cells stably or transiently expressing a DNA copy of the *S1* gene. In stable cell lines containing integrated copies of the engineered *S1* gene, both σ1 and p14 were produced. Synthesis of these proteins was directed by chimeric mRNA consisting of the mouse metallothionein I 5′ leader and part of the rat preproinsulin II 5′ untranslated region, followed by the S1 coding sequence. A series of SV40-derived plasmids containing the reovirus *S1* gene with small sequence changes around the σ1 initiation codon were constructed to study the influence of upstream initiation on second reading frame utilization in mammalian cells. As predicted from the modified scanning model of translation initiation (1), p14 levels increased as the consensus sequence context around the upstream (σ1) AUG was modified. Higher amounts of p14 were also obtained after mutation of the σ1 AUG to UUG, which makes the p14 initiator codon the first AUG in the S1 mRNA. In addition, the level of p14 expression was similarly increased by insertion of an upstream termination codon. Somewhat surprisingly, σ1 levels were not inversely proportional to p14 but apparently remained unchanged in response to variations around the initiation codon (Fig. 5 and ref. 3.), suggesting that σ1 formation is limited not at translation initiation but rather at a subsequent step. That initiation at the σ1 AUG occurs with different efficiencies in the various constructs is strongly suggested by the different amounts of

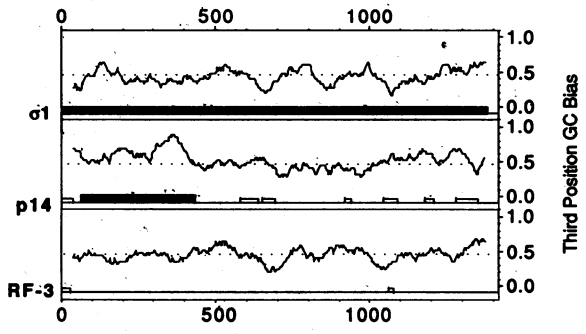

FIG. 6. G+C bias in codons of the S1 reading frames. Third-position G+C bias of reovirus serotype 3 *S1* gene was analyzed as described (28). The reading frames for σ1 and p14 are filled; RF-3 corresponds to the third possible frame that has essentially no coding capacity. The plot above each reading frame represents the G+C content at each third codon position according to the scale on the right. The *x*-axis corresponds to the nucleotide position. The open rectangles are other potential open reading frames.

p14 produced, which probably reflects the number of ribosomes that bypass the first AUG and initiate at the second.

Reinitiation of protein synthesis by eukaryotic ribosomes has been shown to occur after termination of translation of an upstream cistron (25, 26). Translation reinitiation is generally an incomplete process, increasing with the distance between the termination codon and the reinitiating AUG to an optimum of >50% at ≈80 nucleotides (26). However, efficiency of reinitiation in the pCOS-MBN5-derived S1 RNA was almost complete (85% by comparing lanes 4 and 5 in Fig. 5), although the expected value is 10–20% (25), since the distance from the inserted termination codon to the p14 AUG in this construct is 31 nucleotides. This suggests that reinitiation is not the only mechanism responsible for the high level of p14 obtained by introduction of a termination codon after the σ1 AUG.

The data are consistent with the idea that "interference" from ribosomes translating the σ1 reading frame is responsible for a reduction in the rate of p14 synthesis, and when that "interference" is relieved (e.g., by introducing a termination codon), p14 levels increase. The basis for this regulation may lie in the type of codons used to synthesize each protein. Low efficiency of σ1 production, even when translation initiates almost exclusively at the σ1 AUG, suggests that the rate of elongation of σ1 chains is inherently low, possibly due to extensive usage of rare codons. Thus, a ribosome would be expected on average to spend a longer time per codon translating the σ1 reading frame than the p14 frame. In this model, ribosomes translating the p14 frame that are behind a ribosome synthesizing σ1 would move at a slower rate than if there were no σ1 translation. Genes encoding abundant cellular proteins often have a high GC content at the third codon position (27), and analysis of the reovirus type 3 S1 mRNA for third position GC bias (28) predicts abundant expression of p14 (as shown by the curve above the dotted line in the p14 reading frame in Fig. 6). The same type of analysis suggests that there is low translation of σ1 all along the message, including in the segment that overlaps p14 (indicated by the curve mostly below the dotted line in the σ1 reading frame in Fig. 6). This situation can occur whenever a single mRNA is used for the synthesis of two different polypeptides, even if one of the proteins is more efficiently expressed than σ1. The reason is that a ribosome pausing in one reading frame necessarily causes ribosomes in the other frame to pause also, even in regions where they would normally move rapidly. Ribosome pausing may be a reflection of differences in tRNA abundance as already documented in *Escherichia coli, Salmonella typhimurium* and *Saccharomyces cerevisiae* (27). Codon usage could also be involved in the differential sensitivity of various cell types to a particular virus. It should be noted that pausing of translating ribosomes has recently been demonstrated in both wheat germ and rabbit reticulocyte extracts (29).

Ribosome "interference" may have some biological importance for regulating the levels of internal proteins produced from bicistronic mRNAs. It should be possible to test its significance by introducing terminator codons in the σ1 reading frame within the p14 coding region. The effects of these changes on p14 synthesis, analyzed after transfection into COS cells, may provide additional insights into the regulation of protein synthesis in mammalian cells.

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