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Biochemistry

Reovirus hemagglutinin mRNA codes for two polypeptides in overlapping reading frames

(bicistronic mRNA/*S1* gene structure/translation initiation)

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ABSTRACT **Human reovirus s1 mRNA, which codes for the viral hemagglutinin, also directs the synthesis of a previously unrecognized polypeptide of molecular mass 14 kDa in reticulocyte and wheat germ extracts. Hybrid-arrest of translation by selected restriction fragments of cloned *S1* DNA indicated that synthesis of the 14-kDa polypeptide initiates at the second AUG. This was confirmed by NH_2-terminal sequence analyses. The coding sequence for the 14-kDa polypeptide thus lies entirely within the hemagglutinin gene but in a different reading frame. Although not found in virions, the 14-kDa polypeptide apparently is formed in virus-infected mouse L cells, as demonstrated by comparison of [^{35}S]methionine-labeled polypeptides in cell extracts with the corresponding *in vitro* products.**

Eukaryotic mRNAs of both viral and cellular origin are generally monocistronic (1). In most cases the AUG nearest the 5′-terminal cap is exclusively used for initiation of protein synthesis. A "scanning" model based on ribosome binding studies with purified reovirus mRNAs has been proposed to explain these characteristics of eukaryotic initiation (2–4). In this scheme the 40S ribosomal subunit attaches to the messenger 5′ end and scans along the polynucleotide template to the first AUG sequence, which usually corresponds to the translation initiation site.

Later the model was expanded to account for those relatively few mRNAs in which the 5′-proximal AUG triplet is not the exclusive initiator. An extensive literature search led to the recognition of a consensus sequence, R-N-N-A-U-G-(G) (R = purine nucleoside; N = any nucleoside), in the 5′ region of more than 90% of the eukaryotic mRNAs analyzed, including most reovirus mRNA species (5, 6). The consensus sequence in the modified version of the model facilitates initiation by acting as the stop signal for the scanning 40S particle, allowing attachment of the 60S subunit (5, 6). By contrast, in the limited number of mRNAs that contain the first AUG in a context less favorable for initiation—e.g., Y-N-N-A-U-G-(G) (Y = pyrimidine nucleoside), some (but not all) 40S particles fail to stop and continue scanning to the next AUG. By this mechanism a single mRNA could code for more than one polypeptide.

The genome of reovirus type 3 consists of 10 segments of double-stranded RNA that are transcribed by a virion-associated polymerase to produce the corresponding viral mRNAs. Most of these mRNAs contain a single ribosome binding site that includes the 5′-proximal AUG in a consensus sequence, consistent with monocistronic function (7). However, the s1 species of viral mRNA is unusual in that it yielded two unique 80S ribosome-protected fragments. One site included the 5′-proximal AUG in the sequence C-G-G-A-U-G-G, while the other fragment contained the second AUG in an initiation consensus sequence, A-U-A-A-U-G-G in a different reading frame (ref. 8 and Fig. 1). Both AUGs can function as initiator codons, as demonstrated by s1 mRNA-directed formation of the two predicted formylmethionine dipeptides in a reconstituted cell-free system (10). It was therefore of interest to determine if the *S1* gene normally codes for more than a single reoviral product. We report here that purified s1 mRNA directs synthesis of two polypeptides—the virion hemagglutinin σ1 initiated from the first AUG and a previously unrecognized 14-kDa polypeptide that begins at the second. Both polypeptides appear to be formed in mouse L cells infected with type 3 reovirus as well as in cell-free translation systems programmed with s1 mRNA.

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MATERIALS AND METHODS

Synthesis of Reovirus Serotype 3 mRNAs and Selection of the s1 Species. Radiolabeled reovirus mRNA (average specific activity = 3800 cpm/μg) was prepared with type 3 viral cores and [^{3}H]UTP as described (11). s1 mRNA was isolated by hybrid selection with cellulose-bound s1 cDNA cloned in pBR322 (10).

Cell-Free Protein Synthesis and Hybrid Arrest of Translation. *In vitro* translation reactions in either rabbit reticulocyte lysate or wheat germ extract were done as described (12, 13). Protein synthesis assays in the reticulocyte system contained 0.5 μg of reovirus s1 mRNA in a total volume of 50 μl, and incubations were at 30°C for 1 hr. The presence of large amounts of endogenous globin prevented the direct analysis of translation products smaller than 20 kDa in Na$DodSO_4$/polyacrylamide gels. Precipitation with antiserum from a reovirus-infected rabbit was therefore used prior to gel electrophoresis to detect polypeptides in the lower molecular weight range. However, precipitation of a number of polypeptides was not dependent upon the presence of the antiserum. To avoid these complications, a wheat germ system was used for most of the *in vitro* translation experiments. Translation in wheat germ extract was performed with 0.25 μg of s1 mRNA in a 15-μl assay mixture incubated at 25°C for 2 hr. Except where indicated, the final concentration of [^{35}S]methionine (Amersham, 1000–1400 Ci/mmol; 1 Ci = 37 GBq) was 2 μM. Radiolabeled translation products were separated in Na$DodSO_4$/15% polyacrylamide gels (14); after electrophoresis the gels were dried and exposed to x-ray film (Kodak X-Omat AR) for 6–18 hr. Hybrid-arrested translation in wheat germ extracts was carried out as described (15). DNA restriction fragments extracted from 1.2% agarose gels by freeze-thawing the excised crushed gel slices were further purified by passage through a Millipore 0.45-μm filter followed by extraction with phenol. A DNA·mRNA single-stranded ratio of 5:1 was used for the hybridization of reovirus s1 mRNA.

Labeling of Polypeptides in Infected and Uninfected Cells. Mouse L cells growing in suspension culture were concentrated to 1×10^7 cells per ml and infected with reovirus type

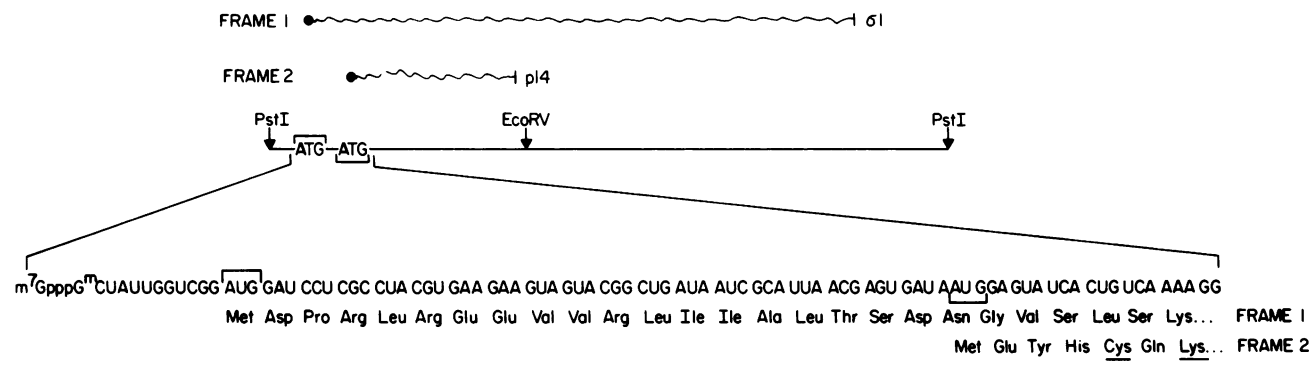

FIG. 1. Structure of reovirus type 3 genome segment *S1* DNA cloned in the unique *Pst* I site of pBR322 (9). The position of the single *Eco*RV cleavage site is indicated relative to the open reading frames for polypeptides σ1 and p14. The 5′-terminal nucleotide sequence of reovirus s1 mRNA was determined by Kozak (7).

3 by adsorption of 10 plaque-forming units per cell for 1 hr at 37°C. Uninfected cells were treated in parallel but without virus addition. The cells were diluted 1:10 in Eagle's minimal essential medium containing L-methionine at 15 mg/liter and 2% fetal bovine serum and incubated for 29 hr at 34°C. Cells were harvested by centrifugation (600 × *g* for 5 min), washed with methionine-free medium, recentrifuged, and resuspended at 10^7 cells per ml in 20 ml of methionine-free Eagle's medium containing 2% fetal bovine serum. [^{35}S]Methionine (2 mCi, 1035 Ci/mmol; Amersham) was added, and incubation was continued for 2 hr at 34°C. Cells were pelleted at 600 × *g* for 5 min, washed with methionine-free medium, resuspended in 5 ml of isotonic salt solution (0.14 M NaCl/0.01 M Tris·HCl, pH 7.5/1.5 mM $MgCl_2$/0.5% Nonidet P-40), and kept on ice for 30 min. The suspension was centrifuged at 400 × *g* for 2 min and the supernatant was frozen and stored at −70°C. Cultures treated with actinomycin D (0.1 μg/ml) were handled similarly except that cells were incubated at 37°C and the drug and [^{35}S]methionine were added at 6 and 15.5 hr after infection, respectively.

Isolation of Polypeptides from Polyacrylamide Gels and Tryptic Peptide Analysis. Radiolabeled polypeptides were recovered from gel slices essentially as described (16). Eluted samples were dialyzed, lyophilized, dissolved in 20 μl of 0.1 M NH_4HCO_3, and incubated with 40 μg of L-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Worthington) at 37°C for 18 hr. Digestion products were oxidized with 200 μl of performic acid for 1 hr at 0°C as described (17), lyophilized, and dissolved in 5 μl of water. The samples were spotted on cellulose sheets and resolved in two dimensions by electrophoresis followed by chromatography as described (18). [^{35}S]Methionine-containing peptides were visualized by autoradiography for 1–4 days.

Protein Sequence Analysis. To determine the positions of cysteine and lysine in the polypeptides synthesized *in vitro*, gel-purified products made in the presence of [^{35}S]cysteine (Amersham; 1460 Ci/mmol) or [^{3}H]lysine (Amersham; 96.3 Ci/mmol) were supplemented with 1 nmol of sperm whale apomyoglobin carrier in 15% (vol/vol) formic acid and analyzed by automated Edman degradation in an Applied Biosystems 470A protein sequencer. Radioactivity recovered at each sequencer cycle was determined by liquid scintillation counting.

RESULTS

Previous studies (8, 10) suggested that the s1 species of reovirus type 3 mRNA might be capable of directing the synthesis of another polypeptide in addition to the viral hemagglutinin that has been mapped to the *S1* gene (19, 20). To test this possibility, s1 mRNA was purified by cDNA-cellulose chromatography and translated in rabbit reticulocyte lysates. The viral mRNA-specified products analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis included a radiolabeled polypeptide of apparent molecular mass of ≈42 kDa (Fig. 2). This is the same value reported for the σ1 hemagglutinin polypeptide component of reovirions (21). A second, more prominent, [^{35}S]methionine-labeled band migrating with lysozyme at ≈14 kDa was also obtained. The cell-free products directed by s1 mRNA in wheat germ extract similarly included a major polypeptide of ≈14 kDa in addition to a 42-kDa band (Fig. 3, lanes 1–3). Apparently there was more premature termination in wheat germ extract than in the reticulocyte lysate system.

Nucleotide sequence analyses of reovirus RNA (7, 8, 22, 23) established that the 5′ end of the s1 mRNA species in-

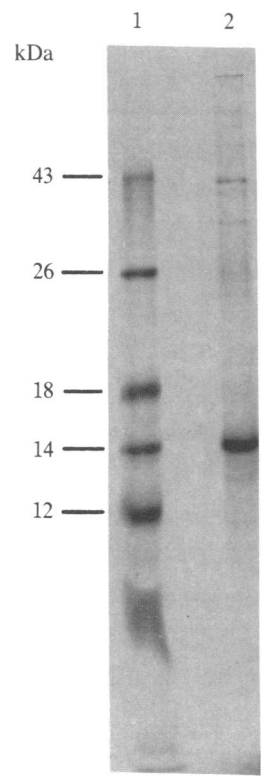

FIG. 2. Cell-free translation of hybrid-selected reovirus s1 mRNA and precipitation of ^{35}S-labeled products. Reticulocyte lysate was incubated in 50-μl translation reaction mixtures with no added mRNA (lane 1) or with 0.5 μg of s1 mRNA (lane 2), and 10-μl samples were precipitated with an equal volume of rabbit antiserum to reovirus type 3 as described (12). Precipitates were analyzed by polyacrylamide gel electrophoresis and autoradiography. ^{14}C-labeled markers and their molecular masses: ovalbumin (43 kDa), α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), and cytochrome *c* (12.3 kDa).

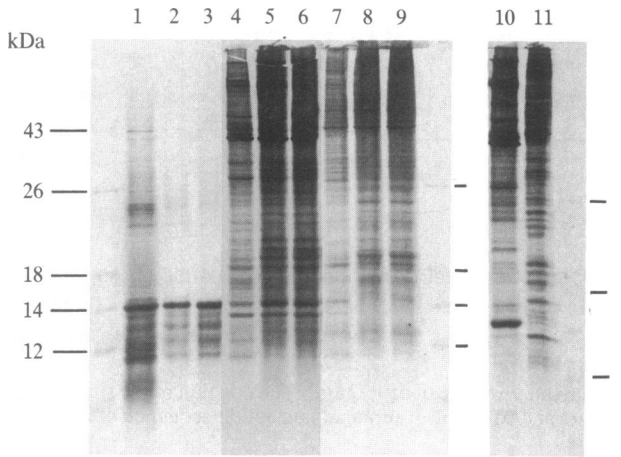

FIG. 3. Translation of s1 mRNA and analysis of ^{35}S-labeled polypeptides from reovirus-infected and uninfected mouse L cells. Hybrid-selected s1 mRNA was translated in wheat germ extract in a 15-μl reaction mixture, and 5-μl aliquots taken before (lane 1) and after precipitation either in the presence of 10 μl of reovirus antiserum (lane 2) or no antiserum (lane 3) were analyzed in a 15% polyacrylamide gel. The same gel was used to analyze extracts of infected (lane 4) and uninfected (lane 7) cells and precipitates of infected cell extract obtained with (lane 5) or without (lane 6) antiserum and the corresponding samples from uninfected extract (lanes 8 and 9). Precipitates, obtained without antiserum, of actinomycin-treated infected (lane 10) and uninfected (lane 11) cell extracts were analyzed in another 15% polyacrylamide gel.

cludes two AUG triplets (Fig. 1). They comprise the central portions of two distinct 80S ribosome binding sites (8). Furthermore, the dipeptides expected from initiation at both AUG triplets—i.e., fMet-Asp and fMet-Glu—were formed *in vitro* in response to template s1 mRNA (10). Recently the complete sequence of a full-length cDNA clone of the *S1* gene of reovirus type 3 has become available (R. Bassel-Duby, A. Jayasuriya, and B. Fields, personal communication). The gene includes two long open reading frames. Frame one extends from the first AUG at residue 13 to a stop codon at a position close to the 3′ end, consistent with the apparent molecular mass of polypeptide σ1. Reading frame 2, which begins at the second AUG, is interrupted by a termination codon after 360 nucleotides—i.e., in good agreement with a polypeptide of apparent molecular mass 14 kDa. To demonstrate that the coding region for the 14-kDa polypeptide coincides with the location of reading frame 2 in s1 mRNA, restriction fragments of s1 DNA were hybridized to the mRNA and tested for arrest of translation.

S1 DNA was excised from pBR322 by digestion with *Pst* I and divided into 5′ and 3′ fragments by cleavage with *Eco*RV (Fig. 1). The translational effects of hybridizing s1 mRNA with these fragments are shown in Fig. 4. The *Pst* I/*Eco*RV fragment that spans the 5′ ends of both reading frames nearly eliminated the template activity of s1 mRNA (lanes 1 and 2). Heat denaturation of the RNA·DNA complexes restored initiation from both AUG codons, as indicated by the synthesis of 42- and 14-kDa products (lane 3). Hybridization of the *Eco*RV/*Pst* I fragment, which lies downstream from reading frame 2, had no inhibitory effect on p14 synthesis (lane 4). However, the yield of the 42-kDa product was markedly diminished, and a new polypeptide was evident at ≈17 kDa (right arrow). This is the size expected for a product resulting from hybrid-arrested translation at or near the *Eco*RV sequence. After heat denaturation (which was apparently incomplete), synthesis of the 42-kDa product was partly restored, and the band at 17 kDa was correspondingly decreased in amount (Fig. 4, lane 5).

The NH_2-terminal sequence of the 14-kDa product was

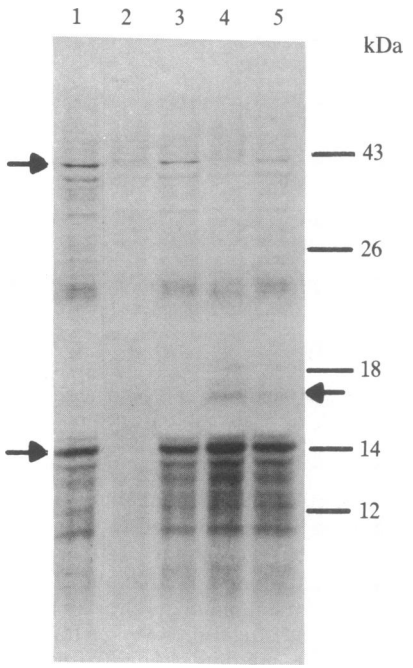

FIG. 4. Hybrid arrest of translation. Reovirus s1 mRNA, hybridized with DNA restriction fragments as indicated, was incubated for 2 hr in the wheat germ translation system, and products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Protein synthesis was assayed with 0.5 μg of mRNA (lane 1), the same amount of mRNA annealed with 1.5 μg of the *Pst* I/*Eco*RV DNA fragment (lane 2) and a duplicate but heated (100°C, 1 min) template (lane 3), or with mRNA hybridized to 3.2 μg of the *Eco*RV/*Pst* I DNA fragment (lane 4) and the corresponding heated sample (lane 5).

also analyzed directly to eliminate any possibility that the polypeptide resulted from premature termination or post-translational processing. The NH_2-terminus predicted for the frame 2 polypeptide includes cysteine at position 5 and lysine at residue 7 (Fig. 1). Stepwise degradation of the 14-

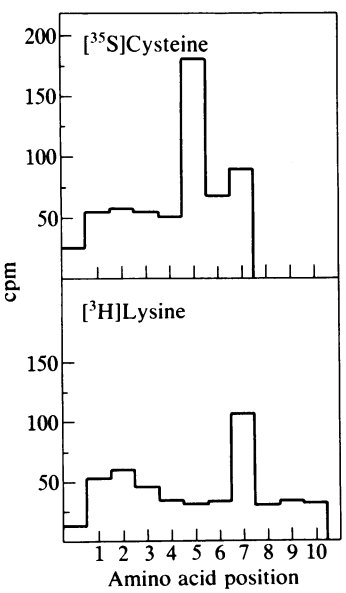

FIG. 5. Automated Edman degradation of polypeptide p14 synthesized in wheat germ and purified by polyacrylamide gel electrophoresis. [^{35}S]Cysteine- (4000 cpm) and [^{3}H]lysine- (6000 cpm) labeled polypeptides were analyzed. Radioactivity recovered at each sequencer cycle is shown.

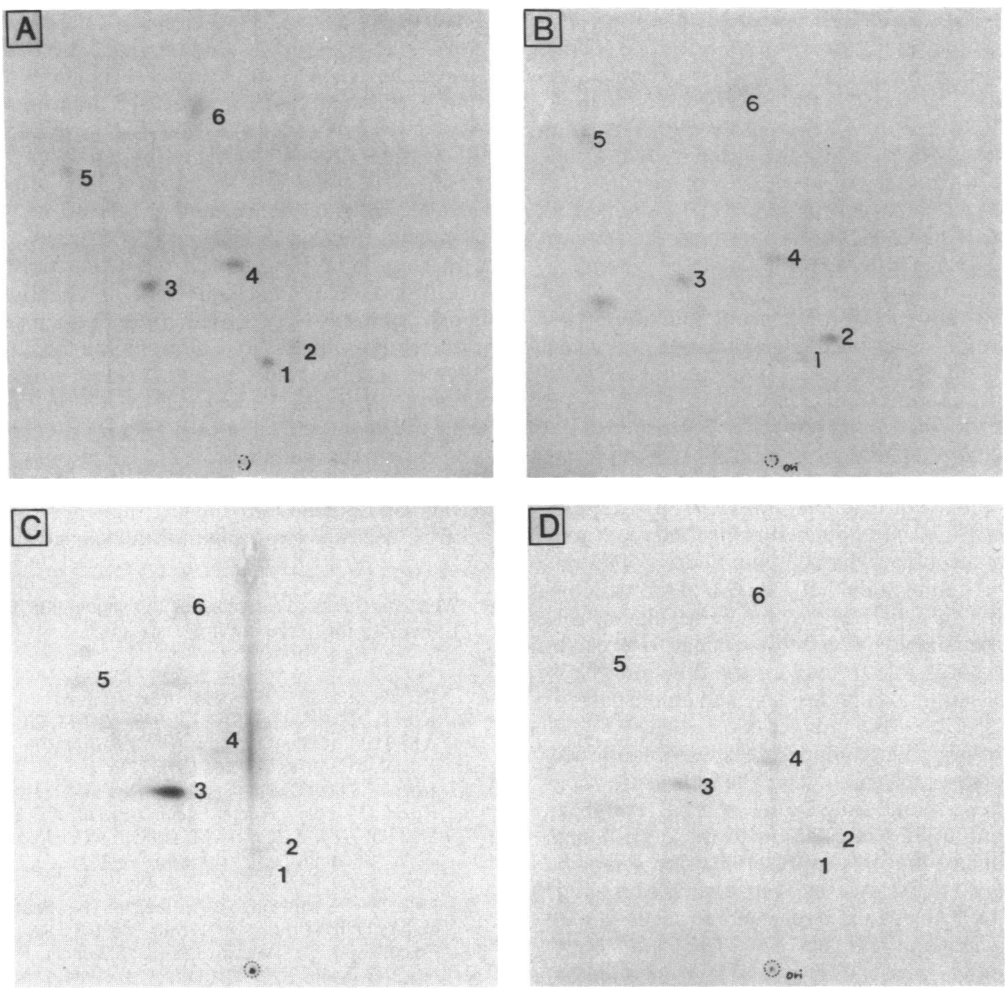

FIG. 6. Tryptic peptide maps of [^{35}S]methionine-labeled polypeptide p14. Products purified by gel electrophoresis were synthesized in wheat germ extract (*A* and *B*, representing two different preparations) or in reovirus-infected L cells as described in *Materials and Methods* (*C*). Equal aliquots of tryptic digests shown in *B* and *C* were mixed for the analysis shown in *D* (20,000 cpm total); 15,000 cpm each was applied to plates *A*, *B*, and *C*. Electrophoresis in the horizontal direction was followed by ascending chromatography (18).

kDa polypeptide synthesized *in vitro* with either [^{35}S]cysteine or [^{3}H]lysine yielded radiolabeled components in exactly the correct positions (Fig. 5).

The results demonstrate clearly that both open reading frames in s1 RNA are translated *in vitro*. It was therefore important to examine type 3 reovirus-infected mouse L cells for the presence of the 14-kDa polypeptide. Extracts of [^{35}S]methionine-labeled infected and uninfected L cells were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The cytoplasmic fraction of infected cells contained many more prominent radiolabeled proteins than the corresponding fraction of uninfected cells (Fig. 3, compare lanes 4 and 7). The infected cell polypeptides (lane 4) included a band that migrated with the *in vitro* p14 product (lane 1). The p14 synthesized *in vitro* was precipitated by incubation with rabbit reovirus type 3 antiserum and centrifugation (lane 2). However, precipitation was not dependent on the presence of antiserum, suggesting that p14 is in readily sedimentable aggregates, possibly complexed with nucleic acids (lane 3). The 14-kDa polypeptide made in infected cells had the same characteristics (lanes 5 and 6). By contrast, the extract of uninfected cells (including the band in lane 7 that migrated slightly slower than the p14 in lane 4) yielded in the pellet fractions no labeled band in the position of p14 (Fig. 3, lanes 8 and 9). Similar results were obtained with cells treated with actinomycin to diminish host protein synthesis—i.e., a prominent 14-kDa polypeptide present in pellets of infected cell extract (lane 10) but not in uninfected extract (lane 11).

To confirm that the 14-kDa polypeptide in the infected cell extract corresponded to the *in vitro* p14 product, the comigrating bands were eluted from polyacrylamide gels, digested with trypsin, and compared by two-dimensional peptide mapping. The p14 synthesized in wheat germ extracts yielded six [^{35}S]methionine-labeled tryptic peptides consistently (Fig. 6*A*), but some samples contained additional spots (Fig. 6*B*). The same amount of radioactive 14-kDa polypeptide from infected cells yielded a similar map that included several corresponding spots (Fig. 6*C*). The higher background may reflect the presence of cellular proteins, and the tendency of the peptides to streak may indicate that p14 is modified in infected cells. Analysis of a mixture containing equal amounts of radioactivity in p14 and infected cell-derived 14-kDa polypeptide indicated an overlap between the tryptic maps, consistent with identity of the polypeptides (Fig. 6*D*). To eliminate the possibility that p14 is coded for by s1 mRNA with a 5′ deletion, primer extension studies were done. The results document a single s1 mRNA species containing both 5′-proximal initiator codons (data not shown).

DISCUSSION

The results demonstrate that the s1 species of reovirus mRNA directs synthesis of two different polypeptides by initiation of translation from both the 5′-proximal and second AUG triplets. The product that begins closer to the mRNA 5′ end is the well-characterized polypeptide σ1, a component

of the outer shell of reovirions. It is responsible for cell attachment and determines reovirus serotype specificity, hemagglutination, and cell and tissue tropism (24). Although important biologically, σ1 is made in relatively minor amounts in virus-infected tissue culture cells or cell-free translation systems and makes up only ≈1% of the virion structural proteins (Fig. 3 and ref. 25). The low frequency of translation of s1 mRNA from the 5′-proximal initiation site is consistent with its sequence, C-G-G-A-U-G-G, in which the initiator codon lies in a context not favored for ribosome binding (5, 6). By contrast, the second initiation site in s1 mRNA includes a consensus sequence for eukaryotic initiation, A-U-A-A-U-G-G; it is effectively used in reticulocyte and wheat germ cell-free protein synthesizing systems and in reovirus-infected cells. The resulting 14-kDa product (p14) represents a previously undescribed reovirus-specific polypeptide. Because its sequence is specified by a different reading frame in s1 mRNA, p14 is probably unrelated in structure or function to polypeptide σ1. For example, unlike σ1, p14 appears to be nonstructural—i.e., not obtained in purified reovirions by silver staining of polyacrylamide gels under conditions that would detect a polypeptide that is 0.05% of the structural proteins (data not shown). p14 may have a regulatory role in the reovirus replication cycle, but this remains to be established. It should be possible to explore the function of p14 with antibodies to synthetic peptides designed on the basis of a hydropathicity profile of the polypeptide.

Utilization of overlapping reading frames was initially recognized as a mechanism for maximizing the information content of a limited-size genome from analyses of bacteriophage φX174 (26). Recent studies in addition to these on human reovirus type 3 suggest that this expression strategy may be employed by many animal viruses. For example, a single mRNA specified by influenza B virus genome segment 6 directs synthesis of the neuraminidase (≈50 kDa) from the second AUG and another polypeptide (≈11 kDa) from an initiator codon only four residues nearer the cap (27). Consequently, the smaller-product reading frame overlaps the larger by 292 nucleotides. A single mRNA from the E1b region of human adenovirus (types 5 and 12) similarly codes for two distinct tumor antigens of ≈19 and ≈54 kDa (28). The open reading frame for the smaller antigen also begins with the first AUG and extensively overlaps the frame for the larger product, starting at the second AUG.

Cell-free translation of influenza bicistronic mRNA (like s1 mRNA) yielded predominantly the smaller product, as compared to similar levels of the two polypeptides in influenza virus-infected cells (27). The possibility that this simply reflects preferential initiation at a 5′-proximal AUG under conditions *in vitro* (27) appears unlikely for reovirus s1 mRNA, which in reticulocyte lysate and wheat germ extract programmed mostly p14, the polypeptide initiated at the second AUG. In reovirus-infected L cells p14 is also apparently a more abundant product than σ1 polypeptide (initiated at the 5′-proximal AUG). This pattern of s1 mRNA translation fits the modified scanning model (5), i.e., a consensus sequence at the second but not the first initiation site. The situation is less clear with respect to the influenza and adenovirus bicistronic RNAs. The relevant influenza virus sequence, A-A-A-*A-T-G*-A-*A*-C-A-*A-T-G*-C-T-A, contains a purine three residues upstream from each of the closely spaced initiating triplets. The initiating ribosome probably spans this entire sequence, and the reading frame selection is influenced by the structure of the complex, charged tRNA availability, and many other factors. In adenovirus E1b mRNA the initiator codons are widely separated; the first occurs in a consensus sequence in adenovirus 12 but not in adenovirus 5, while the second initiation sequence in both types is T-A-A-*A-T-G*-G (28).

Another example of overlapping reading frames expressed via a single mRNA is the template for Sendai virus polypeptides P, an ≈79-kDa component of the nucleocapsid core, and C, an ≈22-kDa nonstructural gene product (29). The former is initiated upstream from the latter in a sequence C-G-C-*A-T-G*-G-A-T-C-A-A-G-*A-T-G*-C—i.e., a suboptimal initiation site followed by a more favorable one as predicted (5). In the Sendai P/C mRNA, as in the reovirus s1 species, the smaller polypeptide initiated at the second AUG is the predominant translation product (29).

Eukaryotic mRNAs that are read in two overlapping reading frames to produce different polypeptides now include single transcripts specified by adeno-, myxo-, paramyxo- and reovirus genes. In addition, the herpes simplex virus thymidine kinase gene encodes related polypeptides by initiation at different 5′-proximal AUGs in the same reading frame in an individual mRNA (30, 31). Recent reports of two *src* proteins in mutant Rous sarcoma virus-transformed rat cells (32) and two proteins encoded by *Saccharomyces cerevisiae GAL4* gene (33) suggest that these mechanisms may also be used for expression of nuclear genes.

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