Characterization of Ribosome Binding on Rous Sarcoma Virus RNA In Vitro

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We determined the sites at which ribosomes form initiation complexes on Rous sarcoma virus RNA in order to determine how initiation of Pr76^{gag} synthesis at the fourth AUG codon from the 5' end of Rous sarcoma virus strain SR-A RNA occurs. Ribosomes bind almost exclusively at the 5'-proximal AUG codon when chloride is present as the major anion added to the translational system. However, when chloride is replaced with acetate, ribosomes bind at the two 5'-proximal AUG codons, as well as at the initiation site for Pr76^{gag}. We confirmed that the 5'-proximal AUG codon is part of a functional initiation site by identifying the seven-amino acid peptide encoded there. Our results suggest that (i) translation in vitro of Rous sarcoma virus virion RNA results in the synthesis of at least two polypeptides; (ii) the pattern of ribosome binding observed for Rous sarcoma virus RNA can be accounted for by the modified scanning hypothesis; and (iii) the interaction between 40S ribosomal subunits or 80S ribosomal complexes is stronger at the 5'-proximal AUG codon than at sites farther downstream, including the initiation site for the major viral proteins.

The factors determining the site(s) at which ribosomes initiate protein synthesis on eucaryotic mRNAs have not been fully elucidated. Although the original scanning hypothesis (15) explained initiation site selection on the majority of eucaryotic mRNAs, a significant number of mRNAs that did not conform to this model remained. These exceptions, in which protein synthesis is initiated downstream from the 5'-proximal AUG codon, include poliovirus (8), Rous sarcoma virus (RSV) (26), and mouse α -amylase (11), which initiate protein synthesis at the ninth, fourth, and second AUG codons from the 5' ends of their RNAs, respectively. Such exceptions led to the formulation of the modified scanning hypothesis, which ranks potential initiation sites by the strength of the sequences flanking the AUG codon (16). The modified scanning hypothesis explains initiation of protein synthesis at sites downstream from 5'-proximal AUG codons by postulating that some 40S ribosomal subunits can bypass weak upstream initiation sites to initiate protein synthesis at the functional AUG codon.

Ribosome-binding studies with RSV RNA have failed to detect ribosome binding at the AUG codon known to initiate synthesis of the *gag* gene product, Pr76^{gag}; rather, binding of ribosomes under conditions precluding peptide bond formation has been detected only within the 5'-proximal 100 bases of RSV RNA with all of the strains tested (4, 23). This observation led to the idea that spatial scanning may occur, in which 40S ribosomal subunits that bind at the 5' end of the RNA can interact with downstream sequences via the secondary structure within the RNA molecule (5, 23). This would allow the 40S ribosomal subunit to bypass upstream AUG codons.

Another explanation for initiation of protein synthesis at downstream AUG codons is that a complete 80S ribosome or its 40S subunit can reinitiate protein synthesis at downstream AUG codons (18, 20) after initiation of protein synthesis at a 5'-proximal AUG codon and synthesis of the encoded peptide. In this model, as in the modified scanning hypothesis, sequences flanking AUG codons may modulate the level of initiation at a given AUG codon.

In a previous report (23) we identified a ribosome-binding site at the 5'-proximal AUG codon on RSV RNA rather than at the initiation codon for Pr76^{gag}. This result was obtained by using a rabbit reticulocyte translational system in which chloride was the principal anion added. However, the results of other studies suggested that initiation by 40S ribosomal subunits is sensitive to chloride and that replacement of chloride with acetate results in enhanced initiation complex formation (27). Furthermore, chloride differentially affects initiation of protein synthesis on some mRNAs; e.g., translation of α -globin mRNA is more sensitive to chloride than translation of β -globin mRNA is (6). We have found that replacement of chloride with acetate as the major anion added to the translational system directed by RSV RNA results in (i) increased synthesis of Pr76^{gag} and (ii) ribosome binding at the first, second, and fourth AUG codons from the 5' end of RSV RNA rather than at only the 5'-proximal AUG codon. In addition, the seven-amino acid peptide encoded behind the 5'-proximal AUG codon is synthesized in translations of RSV RNA, as predicted by the ribosome-binding studies.

MATERIALS AND METHODS

The RNase T1 used for digestion of ribosome initiation complexes was obtained from Calbiochem-Behring. Sequencing grade RNase T1, dinucleotide cap m⁷G5'ppp5'A, and T4 polynucleotide kinase were obtained from P-L Biochemicals, Inc. Leupeptin was obtained from Vega Biochemicals. RSV virion RNA was isolated from chicken embryo fibroblasts infected with RSV strain SR-A as previously described (10). Anisomycin was obtained from Sigma Chemical Co., and sparsomycin was a generous gift from Marilyn Kozak and Matthew Suffness of the National Cancer Institute. [γ -³²P]ATP was obtained from ICN Pharmaceuticals Inc., and [5'-³²P]pCp was obtained from New England Nuclear Corp. High-pressure liquid chromatography (HPLC) grade acetonitrile, trifluoroacetic acid (TFA), and methanol were obtained from Pierce Chemical Co. *n*-Butanol was from Eastman-Kodak Co. The C18 Syncropak column resin used was from Synspec, Inc. The seven-amino acid marker peptide was prepared by Fernando

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FIG. 1. Translation in the presence of either acetate or chloride salts. (A) Translation of RNA isolated from RSV-infected chicken embryo fibroblasts. Lanes 1 and 2, translation in the presence of chloride salts with (lane 1) and without (lane 2) added RNA; lanes 3 and 4, translation in the presence of acetate salts with (lane 3) and without (lane 4) added RNA. (B) Translation of RSV virion RNA. Lanes 1 and 2, translation in the presence of acetate salts with (lane 1) and without (lane 2) added RNA; lanes 3 and 4, translation in the presence of chloride salts with (lane 3) and without (lane 4) added RNA. The major virion RNA-encoded protein products, Pr180^{gag-pol} and Pr76^{gag}, are indicated.

Albreccio and Sam Gunderson (Hackett et al., submitted for publication). The RSV DNA clones used, pSRA5' and pSRA5' subclones 1 through 6, have been described previously (23).

Translation in vitro. The rabbit reticulocyte translation system used was essentially the system described by Pelham and Jackson (22), with some modifications (3) (in addition, no exogenous amino acids were added to the system). RSV RNA was added to a final concentration of 16 to 33 $\mu\text{g}/\text{ml}$ after heat denaturation for 2 min at 80°C in 0.2 mM EDTA. In some experiments potassium acetate and magnesium acetate replaced the chloride salts at final concentrations of 135 mM and 135 μM , respectively. The experiments in which leupeptin was used are described in the figure legends. For inhibition studies the cap analog $m^7\text{G}5'\text{ppp}5'\text{A}$ was used at a final concentration of 0.8 mM. Products of the in vitro translation reactions were separated on 9% polyacrylamide gels with 3% stacking gels by using the procedure of Laemmli (19).

Isolation and analysis of ribosome-protected RNA fragments. Isolation and analysis of the ribosome-protected RNA fragments were performed essentially as described previously (23), with the modifications described below. RNase T1 from Calbiochem-Behring was used to digest ribosome initiation complexes formed in the presence of 3

mM anisomycin or 0.2 mM sparsomycin. Because of the difference in the unit definitions of the manufacturers, using RNase T1 from Calbiochem-Behring resulted in approximately 60-fold-greater digestion than using RNase T1 from Boehringer Mannheim Biochemicals, as demonstrated by the smaller sizes of the oligoribonucleotides recovered after digestion. In addition to using RNA fragments labeled at their 5' ends, RNA fragments were labeled at their 3' ends with ^{32}P after removal of the 3' phosphate left by RNase digestion with calf intestinal phosphatase (CIP), as described by England and Uhlenbeck (9), except that glycerol was omitted from the reaction mixture (7). The hybridization of ^{32}P -labeled, ribosome-protected RNA to cloned RSV DNAs was performed at 34°C, and elution was accomplished by heating the preparation twice at 80°C for 10 min in 0.2 mM EDTA. The eluted RNAs were suspended in a solution containing 7 M urea, 20 mM sodium citrate (pH 5.0), 1 mM EDTA, 0.05% (wt/vol) bromphenol blue, 0.05% (wt/vol) and xylene cyanol and heated for 10 min at 50°C before loading onto a 16.5% polyacrylamide gel. The gel buffer used, elution of specific RNA fragments, and analysis by partial RNase T1 digestion have been described previously (23).

HPLC analysis of translational products. RSV RNA or water was added to a translation mixture containing acetate salts (see above) and 50 μg of leupeptin per ml. After incubation for 4 min at 29°C, a portion of the translation mixture was removed and added to a 0.5-ml microcentrifuge tube containing an equal volume of methanol. After vortexing for 15 s, a volume of *n*-butanol equal to the volume of methanol was added, and the mixture was vortexed for 15 s. More than 99% of the proteins in the lysate were precipitated by cooling at -80°C for ca. 5 min. The supernatant was cleared of the precipitated proteins by centrifugation for 5 min in a 0.5-ml microcentrifuge (Fisher Scientific Co.) and transferred to a fresh microcentrifuge tube. Then 20 nmol of a synthetic marker peptide, H-Met-Ala-Gly-Pro-Leu-Ile-Pro-OH (Hackett et al., submitted), was added, and the samples were dried in a Speed Vac concentrator. Each dried sample was suspended in 30 μl of water, and 20 μl was applied to a C18 column and eluted on a linear 0 to 30% acetonitrile gradient in 0.1% TFA by using a Beckman HPLC apparatus. The HPLC run conditions used were as follows: 0.1% TFA 5 min; a gradient from 0 to 30% acetonitrile in 0.1% TFA for 40 min; and 30% acetonitrile in 0.1% TFA for 5 min. The flow rate was 1 ml/min, and the absorbance was monitored at 210 nm with an LKB Uvicord II detector. [^{35}S]methionine incorporation was detected by collecting 1-ml fractions directly into minivials and adding Tritonol, followed by counting with a Beckman model LS-100C scintillation counter.

RESULTS

Translation of cellular mRNA in rabbit reticulocyte lysates is usually performed by using chloride as the major monovalent anion. Indeed, chloride is more efficient at promoting translation of intracellular mRNA isolated from RSV-infected chicken embryo fibroblasts than acetate is (Fig. 1A). However, since we were unable to detect ribosome binding at the initiation site for Pr76^{gag} on RSV RNA, we replaced chloride with acetate in the translational system. The use of acetate salts instead of chloride salts in the translational system resulted in enhanced translation of RSV RNA (Fig. 1B); the increase was threefold, as measured by the incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble protein.

The increased synthesis of viral proteins when acetate salts were used in the translational system could have been due to an increase in ribosome binding (i) at all ribosome-binding sites or (ii) at specific ribosome-binding sites. Consequently, we examined the sites of ribosome binding under the new salt conditions by using anisomycin to inhibit peptide bond formation in order to determine whether there was detectable binding at the initiation site for Pr76^{gag}, AUG 4 (Fig. 2A). RNA fragments which had been isolated from RNase T₁-digested monosomes were labeled at their 5' ends with ³²P and hybridized to pSRA5' subclones 1 through 6 (Fig. 2A) (23). After elution, the RSV-specific RNA fragments were separated on a 16.5% denaturing polyacrylamide gel (Fig. 2B). The RSV RNA fragments ranged in size from 26 bases (band E) to 35 bases (band D). In contrast to our previous results, which demonstrated ribosome binding only at AUG 1, replacement of chloride with acetate resulted in ribosome binding at AUG 1 (bands A, C, and D), AUG 2 (band B), and AUG 4 (bands E, F, and G). There was no detectable binding to AUG 3; the RNA fragments that hybridized to pSRA5' subclone 3 bound to the region upstream from AUG 3, as reported previously (23). As shown in Fig. 2C, an AUG codon is located at the 5' end of the seven major ribosome-protected RNA fragments. This explains the observation that the RNA fragments generated by protection at AUG 1 hybridized to pSRA5' subclone 2, which contains RSV DNA sequences starting two bases downstream from AUG 1. A comparison of the patterns of ribosome binding obtained with chloride and acetate in the translational system suggested that initiation complex formation at AUG 4 is more sensitive to the anion used than initiation complex formation at AUG 1 is.

We repeated the ribosome protection studies by using a different inhibitor of protein synthesis, sparsomycin, to establish that the new ribosome-binding pattern was not due to the inhibitor but rather to the anion in the translational system. The patterns of ribosome protection when sparsomycin was used in the presence of either chloride or acetate salts are shown in Fig. 3. The presence of chloride salts (Fig. 3A) nearly abolished the formation of initiation complexes downstream from AUG 1 (Fig. 3C). The RNA fragments generated by ribosome binding at AUG 4 could be seen only in long exposures of the gels. In contrast, when acetate salts were used in the translational system (Fig. 3B), easily identifiable initiation complexes were formed at AUGs 2 and 4 in addition to AUG 1 (Fig. 3D), as was the case when anisomycin was used as the inhibitor of protein synthesis. When acetate was used in the translational system with either anisomycin (Fig. 2) or sparsomycin (Fig. 3), ca. 25% of the ribosome-protected RNA fragments hybridized to the region around AUG 4. The major difference in the pattern of ribosome-protected fragments that resulted from the use of sparsomycin was the intense 55-base RSV RNA fragment that hybridized to pSRA5' subclone 1 (Fig. 3A and B, lane 1); there was no comparable protection of this region of RSV RNA when anisomycin was used (Fig. 2B, lane 1, arrow). However, the procedure which we used to label the RNA fragments would not have labeled those fragments that were terminated by a 5' cap structure.

To determine whether any 5' capped RSV RNA fragments were protected by ribosomes, we labeled RNA fragments from the samples used in the experiments shown in Fig. 2 and 3 with ³²P at their 3' ends. The 3'-terminal phosphate groups on the RNA fragments generated by RNase T₁ digestion were removed with CIP before labeling. To facilitate comparison with our previous results and to demon-

strate that the phosphatase reaction had not caused degradation of the RNA, phosphatase-treated and untreated RNAs were labeled at their 5' ends as in our previous experiments. The ³²P-labeled RNA fragments were hybridized to pSRA5' subclone 1, and the RSV RNA fragments were analyzed on 16.5% polyacrylamide gels after elution (Fig. 4). Figure 4 shows the products of 5' labeling reactions performed with untreated (lane 1) and CIP-treated (lane 2) RNA fragments. The patterns of labeling of the fragments in the lanes were not changed, indicating that the CIP treatment did not degrade the RNAs. Lane 3 shows the products of the 3' labeling reaction. Note that the 3' labeling resulted in band shifts due to the addition of one base to the 3' termini; 5' labeling only added a phosphate group to the 5' termini. A comparison of the RSV RNA fragments in lanes 4 and 5, in which fragments were labeled at their 5' ends, with the fragments in lane 6, in which fragments were labeled at their 3' ends, showed that when anisomycin was used in the translational system, a 3'-end-labeled capped RSV RNA fragment of 25 bases was protected (lane 6, arrow). This fragment contained no AUG codons and most likely was the result of a 40S ribosomal subunit stalled at the cap. In contrast, lanes 8 through 10 show a 55-base oligoribonucleotide which could be labeled at both its 5' (lanes 8 and 9) and 3' (lane 10) ends. These results demonstrate that there were no detectable capped RSV RNA fragments protected by ribosomes when sparsomycin was used as the inhibitor of protein synthesis. It is interesting that the capped RNA fragment recovered from reactions containing anisomycin (lane 6) and the 55-base RNA fragment recovered when sparsomycin was used (lane 7) were present in stoichiometric amounts. In both cases these protected RNA fragments probably resulted from preinitiation complexes. Thus, although there were readily apparent differences in the exact sites of protection when the different inhibitors were used, the overall distribution of ribosomes was essentially the same.

Synthesis of Pr76^{gag} is sensitive to inhibition by a variety of cap analogs in reticulocyte lysates (2, 4; Petersen, unpublished data). Therefore, to demonstrate that the ribosome binding which we observed was the result of a bona fide initiation event, we repeated the ribosome-binding experiments by using sparsomycin to inhibit peptide bond formation in the presence or absence of the dinucleotide cap analog m⁷G5'ppp5'A. RNA fragments isolated from RNase T₁-digested monosomes were labeled at their 5' ends with ³²P, and equal numbers of counts from each reaction mixture were hybridized to pSRA5' which contained the *EcoRI*-*Bam*HI fragment shown in Fig. 2A. The eluted RSV RNA fragments were analyzed on a 16.5% polyacrylamide gel (Fig. 5). Addition of the cap analog to the translational system nearly eliminated ribosome binding to RSV RNA when either chloride or acetate salts were used, as determined by the numbers of counts eluted from the pSRA5' filters. This suggests that the ribosome protection shown in Fig. 2 through 4 was the result of ribosome binding to normal initiation sites on RSV RNA.

Although the results described above demonstrated ribosome binding at the initiation site for Pr76^{gag}, ribosomes bound at the 5'-proximal AUG codon under all conditions tested. Productive initiation of protein synthesis at this site would result in the synthesis of a seven-amino acid peptide designated leader peptide 1 (LP1). Consequently, we initiated a search for this peptide in the translational system containing RSV RNA. The results of an early experiment indicated that a dipeptide was degraded rapidly when it was

FIG. 2. Ribosome protection of RSV RNA by using anisomycin and acetate salts in the translational system. (A) Map of the 5' end of RSV strain SR-A RNA and pSRA5' subclones 1 through 6 of the RSV leader region. The map shows the positions of the AUG codons in the leader region of RSV RNA. The stippled regions indicate the open reading frames behind the AUG codons, and the cross-hatched area indicates the *gag* gene coding region. The lower line indicates the subclones of the RSV RNA leader region. E, *Eco*RI; B, *Bam*HI; H, *Hae*III; nt, nucleotide. (B) Distribution of ribosome-protected RSV RNA fragments determined when using acetate salts in the translational system. Lane 0, 32 P-labeled RNA fragments before selection of RSV-specific fragments; lanes 1 through 6, RNA fragments selected by hybridization to pSRA5' subclones 1 through 6, respectively. The arrow indicates the position of the major 55-base RNA fragment which was protected when sparsomycin was used in the translational system (see Fig. 3A and B, lane 1). (C) Map of ribosome protection on RSV RNA in the presence of acetate salts. The ribosome-protected RSV RNA fragments (designated by letters in panel B) were eluted and identified by partial RNase T1 digestion (23). The locations of the fragments are shown with respect to the AUG codons. The hexagons indicate AUG codons (the number in the hexagon refers to the position relative to the 5' end of RSV RNA). The upper lines indicate the distances (in bases) from the 5' end of RSV RNA (note the discontinuity in the scale).

added to the reticulocyte lysate (11), so we tested the ability of the lysate to translate RSV RNA in the presence of leupeptin, a protease inhibitor. Translation of RSV RNA was not measurably inhibited by the presence of leupeptin

up to a concentration of 300 μ g/ml (Petersen, unpublished data).

The seven-amino acid peptide predicted by the nucleic acid sequence was manufactured by solid-phase synthesis

FIG. 3. Ribosome protection of RSV RNA determined when using sparsomycin and either chloride or acetate in the translational system. (A and B) Distribution of ribosome-protected RSV RNA fragments on 16.5% polyacrylamide gels when chloride and acetate salts, respectively, were used. (C and D) Maps of ribosome protection on RSV RNA when chloride and acetate salts, respectively, were used. For additional information see the legend to Fig. 2. nt, Nucleotide.

(1) for use as a marker on analytical HPLC columns, which could separate LP1 from the other peptides in the rabbit reticulocyte translational system (Hackett et al., submitted). The A_{210} profile of marker LP1 extracted from the reticulocyte lysate showed that the peptide eluted at 24 min (Fig. 6) under the gradient conditions described in Materials and Methods. To demonstrate that LP1 was synthesized in translations of RSV RNA, we extracted alcohol-soluble peptides produced in rabbit reticulocyte lysates that had been incubated for 4 min in the presence of RSV virion RNA or water. A peak of [35 S]methionine activity which comigrated with synthetic LP1 was synthesized in translational mixtures containing RSV RNA but not in mixtures containing water (Fig. 6). From this we concluded that LP1 was synthesized in vitro from RSV RNA, as predicted by the ribosome-binding data. On the basis of [35 S]methionine incorporation kinetics during 4-min translations, we calculated that the molar amount of LP1 synthesis was roughly equivalent to the molar amount of Pr76^{aug} synthesis (Petersen, unpublished data). Because of the instability of the peptide in the translational system (Hackett et al., submitted), LP1 could not be detected in translations of RSV RNA performed without added leupeptin (R. Petersen, P. B. Hackett, and S. Gunderson, unpublished data).

DISCUSSION

The current models explaining initiation of protein synthesis on RSV virion RNA include (i) the modified scanning hypothesis, in which 40S ribosomal subunits can bypass weak upstream AUG codons to initiate further downstream, (ii) the spatial scanning hypothesis, in which RNA secondary structure juxtaposes the initiation site with the 5' end of the RNA, and (iii) the reinitiation hypothesis, in which the ribosome or 40S subunit reinitiates downstream after synthesizing a short peptide. Previously, ribosome binding was reported within the 5'-proximal 100 bases of RSV RNA, but there was no evidence of binding at the initiation codon for Pr76^{aug} (4, 23). Consequently, there were no data to support any of the models presented above. We have now identified conditions under which binding at the initiation site for Pr76^{aug} occurs; this allows us to evaluate the applicability of the models to the problem of translation of RSV RNA.

The results of the ribosome-binding assays performed in the presence of acetate salts support the modified scanning hypothesis. Initiation complexes form predominantly at the 5'-proximal AUG codon, with some initiation at downstream sites. Ribosome binding at AUG 4 under conditions that prevent peptide bond formation discounts reinitiation as the

FIG. 4. Detection of capped RSV RNA fragments by 3' ^{32}P end labeling of ribosome-protected RNAs. The RSV RNA fragments labeled with ^{32}P at their 3' ends were isolated by hybridization to and elution from pSRA5' subclone 1; this was followed by analysis on 16.5% polyacrylamide gels. To facilitate comparison with the other experiments (Fig. 2 and 3), parallel 5' ^{32}P -labeling reactions were performed with CIP-treated or untreated RNAs. Lanes 1 through 3, Products of 5' and 3' ^{32}P -labeling reactions performed with the RNA fragments isolated from RNase T_1 -digested initiation complexes before selection for RSV mRNA fragments (lane 1, 5' labeled, no CIP treatment; lane 2, 5' labeled, CIP treated; lane 3, 3' labeled); lanes 4 through 6, RSV-specific RNAs from a reaction in which anisomycin was used as the inhibitor (lane 4, 5' labeled, no CIP treatment; lane 5, 5' labeled, CIP treated; lane 6, 3' labeled after CIP treatment); lanes 7 through 10, RSV-specific RNAs from a reaction in which sparsomycin was used as the inhibitor (lanes 7 and 9, 5' labeled, CIP treated; lane 8, 5'-labeled, no CIP treatment; lane 10, 3' labeled, CIP treated). Lane L was a marker lane containing 5', ^{32}P -labeled, partially alkaline-hydrolyzed poly(A). The arrow indicates the position of the capped RSV RNA fragment protected when anisomycin was used.

only method for initiation at downstream sites on RSV virion RNA. If reinitiation were the major mode by which initiation occurred at downstream sites, we would expect to see ribosome binding mainly at the 5'-proximal AUG codon since reinitiation requires synthesis of LP1 before ribosome movement downstream. In sum, reinitiation should be prevented when peptide bond formation is inhibited. Ribosome binding at AUGs 1 and 2 would not be predicted by the spatial scanning model (5); rather, we would expect binding predominantly at AUG 4. This interpretation must be tempered since the secondary structure that an RNA molecule assumes is undoubtedly dynamic. However, there is a pronounced difference in the pattern of ribosome protection observed that is dependent on the ionic conditions used.

Protein synthesis directed by RNA isolated from RSV-infected chicken embryo fibroblasts is more efficient when

chloride is the major monovalent anion added to the *in vitro* translational system (Fig. 1A). However, protein synthesis directed by RSV RNA is more efficient when acetate replaces chloride as the major monovalent anion added to the translational system, as demonstrated by the enhanced synthesis of Pr76^{gag} (Fig. 1B) and ribosome binding at the initiation site for Pr76^{gag} (Fig. 2 and 3). These results can be interpreted to mean that chloride inhibits initiation complex formation at AUG codons that are not 5' proximal. An alternative explanation is that the use of acetate salts allows nonspecific binding to RNA by ribosomes, thus resulting in multiple initiation sites. The similarity of the translational products shown in Fig. 1 and the specificity of initiation on RSV RNA demonstrated by the m⁷G5'ppp5'A inhibition experiment (Fig. 5) indicate that acetate does not reduce the specificity of initiation. However, there is precedence for

FIG. 5. Effect of cap analog on ribosome protection when sparsomycin and either acetate or chloride salts were used in the translational system. Initiation complexes were formed in the presence or absence of 0.8 mM $m^7G5'ppp5'A$. Isolated RNA was 5' end labeled, and the RSV-specific sequences were selected by hybridization to pSRA5'; for each sample 2×10^8 cpm was hybridized. The RSV-specific RNA fragments were analyzed on 16.5% polyacrylamide gels. Lanes 1 and 2, Initiation complexes formed in acetate salts; lanes 3 and 4, initiation complexes formed in chloride salts; lanes 2 and 4, cap analog added to the translational system.

inhibition of initiation complex formation by chloride in the rabbit reticulocyte translational system.

Weber et al. (27) reported that high concentrations of chloride inhibit initiation of protein synthesis. These authors hypothesized that the high electronegativity of the chloride ion interferes with protein-protein interactions or protein-nucleic acid interactions or both, probably resulting in the dissociation of initiation factors from the 40S ribosomal subunit. This may explain the sensitivity to chloride of initiation complex formation at the initiation codon for Pr76^{gag}. A 40S ribosomal subunit migrating downstream along an RNA molecule may lose initiation factors while in transit, and as a consequence the 40S subunit would not be competent to initiate protein synthesis upon reaching the downstream AUG codons. Alternatively, a 40S ribosomal subunit that reaches a downstream AUG codon in the translational system containing chloride may not form a stable complex; before the complete 80S complex is formed, the 40S subunit may dissociate from the RNA. If the latter explanation is correct, there must be additional factors that stabilize initiation complexes formed at AUG 1.

Initiation complex formation at the initiation site for Pr76^{gag} is more sensitive to the anion added to the translation mixture than initiation at the 5'-proximal AUG codon is. This result is somewhat surprising since analysis of the sequences flanking the two AUG codons (AUG 1, UUGAUGG; AUG 4, AGCAUGG) suggests that AUG 4 is

FIG. 6. HPLC analysis of translation products from RSV-directed or control translations. Translation reaction mixtures were extracted and analyzed with a reverse-phase C18 column as described in Materials and Methods. The line labeled LP1 shows the elution profile of synthetic LP1 monitored by A_{210} . The other lines show the elution profiles of incorporated [35 S]methionine from RSV-directed (●) and control (○) translations.

the stronger initiation site based on the consensus sequence proposed in the modified scanning hypothesis (16). The stronger binding of ribosomes at AUG 1 indicates that the ability of an AUG codon to act as an initiation site depends on features of the RNA other than its immediate flanking sequence. The distance between an AUG codon and the 5' terminus of the mRNA may be important (18). In this context we note that AUG 1 is 41 nucleotides from the 5' end of the RNA molecule; this is within the typical range of cap-to-initiation site distances found on most eucaryotic mRNAs (17). AUG 4 is 331 nucleotides farther downstream. Thus, the relative insensitivity of ribosome binding at AUG 1 to the anion present in the translation mixture may result from the proximity of this site to the cap. Preliminary ribosome-binding studies with uncapped RSV RNAs synthesized *in vitro* showed a reversal of binding strengths, with AUG 4 being stronger than AUG 1 (R. Petersen and C. Hensel, unpublished data). This suggests that the cap structure at the 5' terminus of RSV RNA enhances ribosome binding to the 5'-proximal AUG codon by conferring stability to the initiation complex formed there.

The observation that ribosome binding occurs more frequently at AUG 1 than at AUG 4 is difficult to explain since AUG 4 is used to initiate synthesis of the major viral proteins Pr76^{gag}, Pr180^{gag-pol}, and gp90^{env}. One possible explanation is that while the 5'-proximal 374 bases of RSV RNA contains the initiation site for viral protein synthesis, it also contains the sequences necessary to initiate viral replication (the tRNA^{Trp} binding site [12, 13, 25]) and to facilitate viral RNA packaging (24). The relative instability of the interaction between the 40S ribosomal subunit or the 80S ribosome and RSV RNA beyond AUG 1 which was demonstrated in the

chloride-versus-acetate experiments may reflect a means of regulating these different functions. Weak binding by 40S ribosomal subunits may facilitate cDNA synthesis by allowing the polymerase to displace the 40S subunit from the RNA. Alternatively, initiation complex formation at AUG 1 may prevent viral proteins from associating with the RNA. A third possibility is that initiation complex formation at AUG 1 may keep the RNA free of ribosomes and available for packaging or cDNA synthesis. Consequently, although we have demonstrated that the ribosome binding which we observe at AUG 1 is functional by isolating LP1 from translational reactions containing RSV RNA, LP1 may not be important in the viral life cycle; rather, initiation by ribosomes at AUG 1 may be a regulatory event in and of itself (Hackett et al., submitted).

Although our data suggest that the modified scanning hypothesis adequately explains initiation of Pr76^{gag} synthesis, this model does not account for the synthesis of all of the proteins encoded by the RSV genome. After splicing to produce *src* mRNA (10), AUG 4 is followed by a termination codon, and an AUG codon downstream from the 3' splice site is used to initiate pp60^{src} synthesis. Two recent reports (14, 21) have indicated that multiple AUG codons are probably functional on *src* mRNA. Consequently, reinitiation may play a major role in the synthesis of pp60^{src}.

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LITERATURE CITED

- Barany, G., and R. B. Merrifield. 1979. Solid-phase peptide synthesis, p. 1-150. In E. Gross and J. Meienhofer (ed.), *The Peptides: Analysis, synthesis and biology*, vol. 2. Academic Press, Inc., New York.
- Beemon, K., and T. Hunter. 1977. *In vitro* translation yields a possible Rous sarcoma virus *src* gene product. *Proc. Natl. Acad. Sci. USA* 74:3302-3306.
- Brandt, T. L., and P. B. Hackett. 1983. Characterization of messenger RNA by direct translation of mRNA from agarose gels. *Anal. Biochem.* 135:401-408.
- Darlix, J.-L., P.-F. Spahr, P. A. Bromley, and J.-C. Jaton. 1979. *In vitro*, the major ribosome binding site on Rous sarcoma virus RNA does not contain the nucleotide sequence coding for the N-terminal amino acids of the *gag* gene product. *J. Virol.* 29:597-611.
- Darlix, J.-L., M. Zuker, and P.-F. Spahr. 1982. Structure-function relationship of Rous sarcoma virus leader RNA. *Nucleic Acids Res.* 10:5183-5196.
- Di Segni, G., H. Rosen, and R. Kaempfer. 1979. Competition between α - and β -globin messenger ribonucleic acids for eucaryotic initiation factor 2. *Biochemistry* 18:2847-2854.
- Donis-Keller, H. 1979. Site specific enzymatic cleavage of RNA. *Nucleic Acids Res.* 7:179-192.
- Dorner, A. J., L. F. Dorner, G. R. Larsen, E. Wimmer, and C. W. Anderson. 1982. Identification of the initiation site of poliovirus polyprotein synthesis. *J. Virol.* 42:1017-1028.
- England, T. E., and O. C. Uhlenbeck. 1978. 3'-Terminal labelling of RNA with T4 RNA ligase. *Nature (London)* 275:560-561.
- Hackett, P. B., R. Swanstrom, H. E. Varmus, and J. M. Bishop. 1982. The leader sequence of the subgenomic mRNA's of Rous sarcoma virus is approximately 390 nucleotides. *J. Virol.* 41:527-534.
- Hagenbuchle, O., R. Bovey, and R. A. Young. 1980. Tissue-specific expression of mouse α -amylase genes: nucleotide sequence of isoenzyme mRNAs from pancreas and salivary gland. *Cell* 21:179-187.
- Haseltine, W. A., D. G. Kleid, A. Panet, E. Rothenberg, and D. Baltimore. 1976. Ordered transcription of RNA tumor virus genomes. *J. Mol. Biol.* 106:109-131.
- Haseltine, W. A., A. M. Maxam, and W. Gilbert. 1977. Rous sarcoma virus genome is terminally redundant: the 5' sequence. *Proc. Natl. Acad. Sci. USA* 74:989-993.
- Hughes, S., K. Mellstrom, E. Kosik, F. Tamanai, and J. Brugge. 1984. Mutation of a termination codon affects *src* initiation. *Mol. Cell. Biol.* 4:1738-1746.
- Kozak, M. 1978. How do eucaryotic ribosomes select initiation regions in messenger RNA? *Cell* 15:1109-1123.
- Kozak, M. 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eucaryotic ribosomes. *Nucleic Acids Res.* 9:5233-5252.
- Kozak, M. 1984. Compilation and analysis of sequence upstream from the translational start site in eucaryotic mRNAs. *Nucleic Acids Res.* 12:857-872.
- Kozak, M. 1984. Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin *in vivo*. *Nature (London)* 308:241-246.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
- Liu, C.-C., C. C. Simonsen, and A. D. Levinson. 1984. Initiation of translation at internal AUG codons in mammalian cells. *Nature (London)* 309:82-85.
- Mardon, G., and H. E. Varmus. 1983. Frameshift and intragenic suppressor mutations in a Rous sarcoma provirus suggest *src* encodes two proteins. *Cell* 32:871-879.
- Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translational system from reticulocyte lysate. *Eur. J. Biochem.* 67:247-256.
- Petersen, R. B., C. H. Hensel, and P. B. Hackett. 1984. Identification of a ribosome-binding site for a leader peptide encoded by Rous sarcoma virus RNA. *J. Virol.* 51:722-729.
- Shank, P. R., and M. Linial. 1980. Avian oncovirus mutant (SE21Q1b) deficient in genomic RNA: characterization of deletion in the provirus. *J. Virol.* 36:450-456.
- Shine, J., A. P. Czernilofsky, R. Friedrich, J. M. Bishop, and H. M. Goodman. 1977. Nucleotide sequence at the 5' terminus of the avian sarcoma virus genome. *Proc. Natl. Acad. Sci. USA* 74:1473-1477.
- Swanstrom, R., H. E. Varmus, and J. M. Bishop. 1982. Nucleotide sequence of the 5' noncoding region and part of the *gag* gene of Rous sarcoma virus. *J. Virol.* 41:535-541.
- Weber, L. A., E. D. Hickey, P. A. Maroney, and C. Baglioni. 1977. Inhibition of protein synthesis by Cl⁻. *J. Biol. Chem.* 252:4007-4010.