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Internal Initiation of Translation on the Vesicular Stomatitis Virus Phosphoprotein mRNA Yields a Second Protein

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In vitro translation of a mixture of the vesicular stomatitis virus (VSV) polyadenylated mRNAs yielded a previously undetected protein with a molecular weight of approximately 7,000 (7K protein). Hybrid-arrested translation demonstrated that both the 7K protein and the VSV phosphoprotein (P protein) were encoded by the P protein message. Immunoprecipitation of the 7K protein with monoclonal antiserum directed against the P protein indicated that the two products were encoded in the same open reading frame. A protein of approximately the same size was immunoprecipitated from cytoplasmic extracts of VSV-infected cells by both the polyclonal and monoclonal antisera, and it is likely that it was a previously unrecognized viral gene product. Translational mapping of the P protein mRNA in vitro indicated that the 7K protein was encoded in the 3′ one-third of the sequence. The synthesis of the 7K protein in vitro was unaffected by hybrid arrest conditions which blocked the 5′ two-thirds of the mRNA and inhibited synthesis of the P protein. These results imply that the ribosomes bind and initiate translation internally on the P protein mRNA at a site located hundreds of nucleotides downstream from the capped 5′ end.

The genome of vesicular stomatitis virus (VSV), the prototype of the rhabdoviruses, is a single-stranded RNA chain with a molecular weight of approximately 3.6×10^6. The genome is complementary to the viral-specific mRNAs, and hence VSV is classified as a negative-strand virus (38). VSV encodes five separate capped, methylated, polyadenylated mRNAs which are synthesized in vivo and in vitro by the virion-associated transcriptase (1, 2, 23, 24). Although the mechanism for transcription remains controversial (7, 36), it has been suggested that the mature mRNAs are monocistronic and that each encodes only a single polypeptide chain. The products have been designated nucleocapsid (N) protein, phosphoprotein (nonstructural [NS] protein, hereafter designated P protein), matrix (M) protein, glycoprotein (G protein), and polymerase (L protein).

Recent work in several laboratories has shown that certain mRNAs from other nonsegmented and segmented negative-strand viruses are polycistronic because they direct the synthesis of more than one protein. These may be encoded by the same open reading frame (ORF) (5, 27, 35), by overlapping ORFs (3, 4, 11), or by spliced transcripts (17). In several cases, the polycistronic mRNA is encoded by the P protein gene (3, 5, 11, 27, 35). However, to date, only a single protein product of the VSV P protein gene has been identified in the virion or in extracts of VSV-infected cells. The possibility that more than one product may be encoded by the P protein gene has not been formally addressed until now. The results that I present here indicate that the VSV P protein mRNA directs the synthesis of a second product, in addition to the P protein, by initiating translation at an internal AUG codon.

MATERIALS AND METHODS

Viral mRNA. VSV mRNA was synthesized in vitro in a reaction mixture containing (final concentration) Tris hydrochloride (pH 8.0) (50 mM), NaCl (0.1 M), dithiothreitol (4 mM), $MgCl_2$ (5 mM), ATP, CTP, and GTP (1 mM each), UTP (0.1 mM), [^{3}H]UTP (50 μCi/ml), RNasin (Promega; 1 U/μl), *S*-adenosylmethionine (1 mM), Triton N-101 (0.05%), and VSV-Indiana Mudd/Summers (25) (100 μg/ml); the reaction mixture was incubated for 5 h at 30°C. The RNA was extracted with phenol-chloroform and chromatographed through Sephadex G-50. Polyadenylated sequences were selected on oligo(dT)-cellulose.

Intracellular mRNA was extracted at 4.5 h after infection of suspension BHK cells with the Mudd/Summers isolate of VSV-Indiana. The cells were disrupted with 1% Nonidet P-40 (NP-40) in hypotonic buffer. The extract was adjusted to contain 0.1% sodium deoxycholate, and the nuclei were pelleted by low-speed centrifugation. The supernatant was layered onto a 5% sucrose shelf prepared in 0.1 M NaCl–0.05 M Tris hydrochloride (pH 7.5)–5 mM EDTA over a preformed 20 to 40% CsCl gradient in TE buffer (1 mM Tris hydrochloride [pH 7.5]–1 mM EDTA) and centrifuged at 37,000 rpm for 18 h in the SW41 rotor (19). The RNA was recovered from the gradient pellet, extracted with phenol-chloroform, and chromatographed through oligo(dT)-cellulose. Agarose gel electrophoresis and blot analysis were performed as previously described (13, 18, 29, 37). ^{32}P-labeled mRNA was prepared and nuclease mapping was performed as previously described (13).

The *Hin*cII A fragment of the pNS319 cDNA insert (9) was subcloned into the *Hin*cII site of plasmid pSP64 (21). After linearization at the unique *Bam*HI site, the plasmid was transcribed in vitro by using the bacteriophage SP6 RNA polymerase (Promega) under the conditions recommended by the supplier. The RNA was extracted and purified as outlined above, but without oligo(dT)-cellulose chromatography.

Hybrid arrest. mRNA was hybridized to a three- to fivefold molar excess of single-stranded VSV-specific cDNA cloned into bacteriophage M13 in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.5) (20 mM)–NaCl (0.4 M) at 65°C for 20 min. The nucleic acids were precipitated with ethanol and suspended in sterile water just before the addition of the other components of the translation reaction mixture.

In vitro translation. mRNA (50 to 150 ng) was translated in

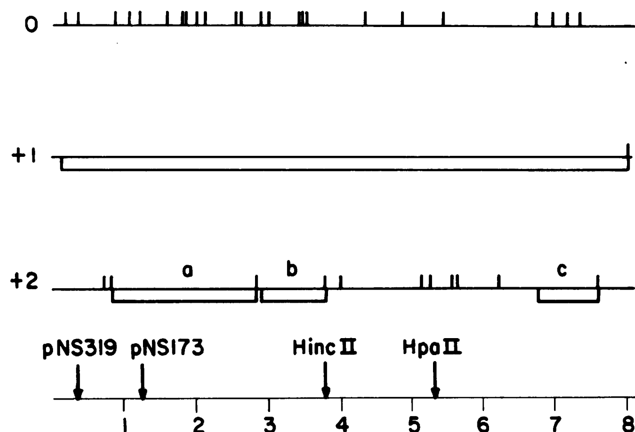

FIG. 1. Coding potential of the P protein mRNA. The coding potential of each reading frame in the P protein mRNA is shown with the 5′ end of the plus sense sequence at the left. Potential open reading frames initiated with an AUG codon are indicated by the open bars; termination codons are indicated by the short vertical lines (based on the data of Gallione et al. [9] as amended by Rose [personal communication]). The locations of the *Hpa*II and *Hinc*II restriction sites and the 5′ ends of the pNS173 and pNS319 clones are indicated on the scale at the bottom, which is marked in increments of 100 nucleotides.

a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) containing (final concentrations) RNasin (1 U/μl), [^{35}S]methionine (1 μCi/μl), magnesium acetate (0.7 mM), potassium acetate (98 mM), and amino acids minus methionine (0.02 mM each) in a final volume of 25 to 50 μl at 30°C for 2 h; the lysate contributed 70% of the total volume. Several different lots were used with equivalent results.

Virion and intracellular proteins. Labeled virions were purified from the growth medium of suspension BHK cells that had been incubated with [^{35}S]methionine (50 μCi/ml) between 2 to 5.5 h postinfection. The virions were pelleted onto glycerol for 2 h at 30,000 rpm in the SW41 rotor and then banded to equilibrium in a 7 to 52% sucrose gradient in TE buffer. The virus band was recovered from the sucrose and stored at −70°C in TE buffer plus 10% dimethyl sulfoxide.

BHK cells were harvested at 5.5 h postinfection and disrupted in hypotonic buffer containing the protease inhibitors (final concentration) phenylmethylsulfonyl fluoride (PMSF) (2 mM), leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), and aprotinin (400 U/ml) by the addition of NP-40 (1%) and sodium deoxycholate (0.5%). Nuclei were pelleted, and cytoplasmic extracts were stored at −70°C. Mock-infected cells were treated in parallel.

Immunoprecipitation. Portions of the reticulocyte lysates were adjusted to contain 50 mM Tris hydrochloride (pH 7.4), 0.15 M NaCl, 5 mM EDTA, and 0.05% NP-40, with PMSF (1 mM) and 1,10-phenanthroline (0.1 mM) added as protease inhibitors. Two different preparations of polyclonal rabbit antiserum (provided by R. A. Lazzarini and S. A. Moyer) were used in these experiments with similar results. After incubation with the antiserum for 1 h at room temperature, fixed *Staphylococcus aureus* (14) was added and the incubation was continued for 30 min. The adsorbed immunoprecipitates were pelleted in a microfuge for 30 s, washed six times in buffer containing PMSF (1 mM), suspended in gel sample buffer, and boiled for 4 min.

Where indicated, mouse monoclonal anti-P protein antibody (provided by M. Williams and S. U. Emerson) was added, and the samples were incubated at room temperature for 1 h. Excess polyclonal rabbit antiserum directed against mouse immunoglobulins (Miles Laboratories, Inc.) was then added, and incubation was continued for an additional hour. Fixed *Staphylococcus aureus* protein was added, and sample preparation was continued as outlined above.

Equal portions of the cytoplasmic extracts were adjusted to contain 6.2 M urea in 50 mM Tris hydrochloride (pH 7.4)–5 mM EDTA on ice and then diluted such that the final concentrations were 0.28 M urea, 50 mM Tris hydrochloride (pH 7.4), 0.15 M NaCl, 5 mM EDTA, 0.05% NP-40, 1 mM PMSF, 0.5 μg of leupeptin per ml, and 0.7 μg of pepstatin per ml. Either polyclonal rabbit or monoclonal mouse antiserum was added, and the samples were immunoprecipitated as outlined above.

Gel electrophoresis. All protein samples were electrophoresed in 17.5% polyacrylamide gels containing sodium dodecyl sulfate (SDS) (16) until the bromophenol dye reached the bottom. The gels were fixed with acetic acid in methanol and then impregnated with Amplify (Amersham Corp.). The dried gels were exposed against Kodak X-Omat AR film at −70°C.

RESULTS

The coding potential of the P protein mRNA sequence from the San Juan isolate of VSV-Indiana published by Gallione et al. (9) as amended by J. K. Rose (personal communication) is illustrated in Fig. 1. The 0 reading frame is extensively blocked by termination codons and does not contain a single initiation codon. The +1 reading frame contains an initiation codon at positions 11 to 13 from the 5′ end of the mRNA and a termination codon at positions 807 to 809. This ORF could encode a protein containing 265 amino acids and having a predicted molecular weight of 29,874. The product of this ORF is likely to be the P protein (9).

The +2 reading frame contains several small potential ORFs. The first, ORF+2a, begins with an AUG at positions 84 to 86 from the 5′ end of the mRNA and terminates with a single UAG codon at positions 285 to 287. It could encode a protein containing 67 amino acids and having a molecular weight of approximately 8,000. ORF+2b begins with an AUG at positions 288 to 290 and is separated from ORF+2a by only the one termination codon. ORF+2b is open for 31 triplets and could encode a protein with a molecular weight of about 3,700. ORF+2c begins at positions 675 to 677. It could encode a protein containing 28 amino acid residues with a molecular weight of approximately 3,100.

Translation of in vitro mRNA. For the experiments reported here, I used the mixture of polyadenylated VSV-specific mRNAs that was synthesized in vitro by the Mudd/Summers isolate of VSV-Indiana (25) as the template for protein synthesis. The purified mRNAs were translated in vitro in a nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S]methionine and placental RNase inhibitor (RNasin). The viral-specific products were immunoprecipitated with polyclonal rabbit anti-VSV antiserum and *Staphylococcus aureus* protein A (14) in the presence of two protease inhibitors (see Materials and Methods). They were subsequently analyzed in a 17.5% polyacrylamide gel containing SDS (16). Under these conditions, the A and B chains of insulin (molecular weights of 2,300 and 3,400, respectively) remained in the gel.

The in vitro mRNAs directed the synthesis of several products which comigrated with proteins found in purified VSV virions (Fig. 2, lanes a and c). Three of these products were identified on the basis of their mobilities as the VSV N,

P, and M proteins. Little or no viral G or L protein was detected because of the small amounts of those messages produced in vitro, and at least in the case of G protein, because it is normally synthesized by membrane-bound polysomes (22).

In addition to these three known proteins, a prominent product with a molecular weight of approximately 7,000 to 8,000 was also synthesized (Fig. 2, lane c). A protein of this size was not detected after immunoprecipitation of the products synthesized by the lysate in the absence of exogenous viral mRNA (Fig. 2, lane b), and it was therefore likely to be VSV encoded. Numerous different preparations of mRNA synthesized in vitro or in vivo by the Mudd/Summers isolate yielded a similar translation product. Identical results were also obtained with poly(A)$^+$ mRNA (provided by M. Hunt) that was synthesized in vitro by the Glasgow isolate of VSV-Indiana (data not shown). RNase digestion of the reaction mixtures before immunoprecipitation did not affect the mobility or recovery of the 7,000-molecular-weight product (7K product) (data not shown).

Identification of the mRNA encoding the 7K protein. Hybrid-arrested translation (26) was used to determine which VSV mRNA encoded the 7K product. The mixture of in vitro messages was hybridized to an excess of single-stranded DNA obtained from a bacteriophage M13 recombinant which carried the minus sense strand of the cloned VSV-San Juan cDNA (9, 32). The mRNAs were subsequently translated in the reticulocyte lysate, and the immunoprecipitated products were analyzed by gel electrophoresis.

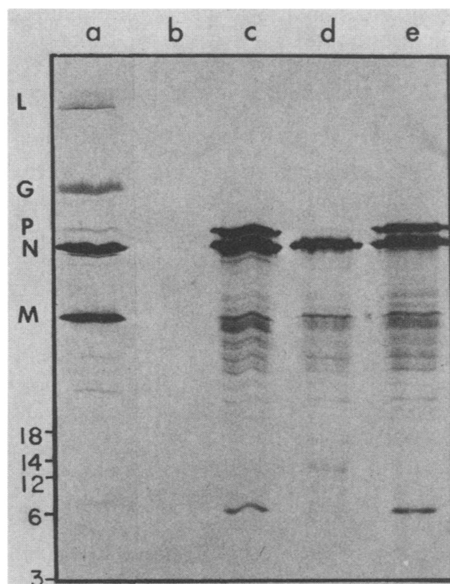

FIG. 2. In vitro translation of mRNA. Poly(A)$^+$ mRNA (85 ng) synthesized in vitro by the Mudd/Summers isolate of VSV-Indiana was translated in a reticulocyte lysate as described in Materials and Methods. Equal proportions of the products were immunoprecipitated and applied to a 17.5% polyacrylamide gel containing SDS. Lanes: a, labeled virion proteins, identified at left, not immunoprecipitated; b, translation without exogenous viral mRNA; c, VSV mRNA not incubated before translation; d, mRNA hybrid arrested with approximately 0.5 pmol of the minus sense strand of the pNS173 insert cloned into bacteriophage M13; e, mRNA hybrid arrested with approximately 0.5 pmol of the plus sense strand of the pNS173 insert cloned into M13. The locations of protein molecular weight standards, detected by Coomassie blue staining, are indicated as follows: 3, unresolved insulin A and B chains (2,300 and 3,400, respectively); 6, bovine trypsin inhibitor (6,200); 12, cytochrome *c* (12,300); 14, lysozyme (14,300); 18, β-lactoglobulin (18,400).

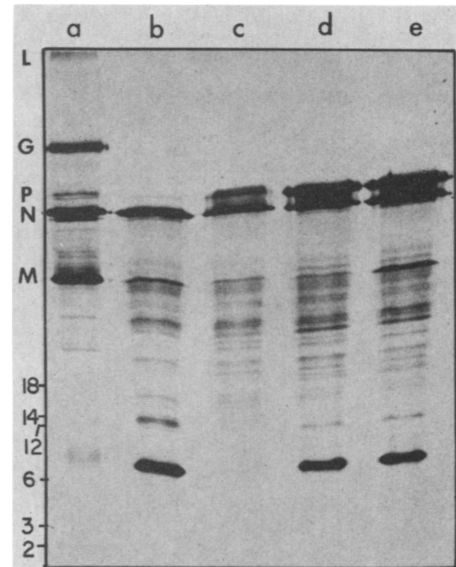

FIG. 3. Translation after hybrid arrest with *Hpa*II fragments of pNS173. In vitro mRNA (60 ng per reaction) was translated in each lysate; hybrid arrest was as indicated, with approximately 0.35 pmol of pNS173-derived insert cloned into bacteriophage M13. Equal proportions were immunoprecipitated and electrophoresed. Lanes: a, virion proteins, identified at left; b, hybrid arrest with the minus sense *Hpa*II A fragment; c, hybrid arrest with the minus sense *Hpa*II B fragment; d, hybrid arrest with the plus sense *Hpa*II B fragment; e, mRNA not incubated before translation. The locations of protein molecular weight standards are indicated as explained in the legend to Fig. 2.

Hybridization to M13 DNA containing the P-protein-specific minus sense strand of plasmid pNS173 (32) simultaneously abolished the synthesis of both the P and 7K proteins without affecting the synthesis of the N or M protein (Fig. 2, lane d). Hybridization of the mRNAs to an equivalent amount of single-stranded DNA from a clone carrying the plus sense strand of the pNS173 insert had no effect on translation (Fig. 2, lane e). These results indicated that the hybrid arrest of P and 7K protein synthesis required the addition of minus sense P-protein-specific cDNA and therefore that both proteins were encoded by a common VSV-specific mRNA.

Localization of the sequence encoding the 7K protein. To localize the region of the P protein mRNA which encodes the 7K protein, hybrid-arrested translations were performed with the *Hpa*II fragments of the pNS173 insert which were also subcloned into bacteriophage M13. The minus sense strand of the *Hpa*II A fragment hybridized to nucleotides 127 to 533 of the P protein mRNA sequence, and the minus sense strand of the *Hpa*II B fragment hybridized to nucleotides 534 to 814 of the sequence (Fig. 1).

As expected, hybrid arrest with the minus sense strand of the *Hpa*II A fragment inhibited the synthesis of the P protein (Fig. 3, lane b). However, this fragment did not affect, or perhaps enhanced, the synthesis of the 7K protein. Hybrid arrest with the minus sense strand of the *Hpa*II B fragment severely reduced the synthesis of the P protein relative to

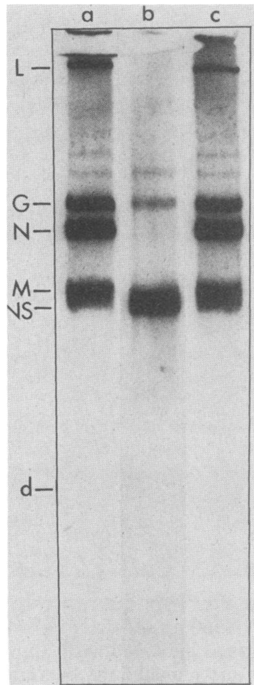

FIG. 4. Nitrocellulose blot analysis of in vivo mRNA. [^{3}H]uridine- and ^{32}P-labeled poly(A)$^+$ mRNA was isolated from VSV-infected BHK cells at 4.5 h postinfection as outlined in Materials and Methods. Portions of each preparation were electrophoresed in a 20-cm, 2% agarose gel containing 6 M urea at pH 3.0 (18) until the bromophenol blue dye migrated approximately 12 cm. The RNA was blotted onto nitrocellulose and then hybridized with the nick-translated insert from plasmid pNS173. In vitro translation confirmed that this ^{3}H-labeled mRNA preparation directed the synthesis of the 7K protein (not shown). Lanes: a and c, uniformly ^{32}P-labeled in vivo mRNA, identified at left; b, ^{3}H-labeled in vivo mRNA. The location of the tracking dye (d) is indicated.

synthesis in the control (Fig. 3, lanes c and e) and abolished the synthesis of the 7K protein. Hybrid arrest with the plus sense strand of the *Hpa*II B fragment had no effect on translation (Fig. 3, lane d).

Identical results for hybrid-arrested translation were also obtained with purified total poly(A)$^+$ mRNA that was extracted from BHK cells infected with the Mudd/Summers isolate of VSV-Indiana. The 7K protein was synthesized even when the 5′ two-thirds of the in vivo P protein mRNA was completely blocked (data not shown). Nuclease mapping experiments, analyzed under two different sets of conditions, indicated that the two M13 *Hpa*II subclones hybridized to the appropriate regions of the P protein mRNA (data not shown). These experiments confirmed that the P and 7K proteins were both encoded by the P protein mRNA. However, the data indicated that the smaller product was translated from the 3′ one-third of that message and not from ORF+2a located near the 5′ end.

Blot analysis of mRNA. Although splicing of the VSV mRNAs has not been reported, previously published experiments may not have detected a small transcript capable of encoding a 7K polypeptide. To address this question, I performed a Northern blot analysis (37) on in vivo poly(A)$^+$ mRNA that was electrophoresed under conditions in which a 200-nucleotide transcript would have migrated only about one-half of the length of an agarose-urea gel (18). After transfer to nitrocellulose, the RNA was hybridized to the nick-translated insert isolated from plasmid pNS173.

The smallest discrete RNA detected by the probe comigrated with the P protein message present in a mixture of uniformly ^{32}P-labeled intracellular poly(A)$^+$ viral species (Fig. 4). Two low-abundance species that were larger than the P protein mRNA were also detected. One of these comigrated with G protein message and was probably the previously identified P-M readthrough product (12); the other was probably an N-P readthrough transcript (12, 20). These results suggested that the 7K product was encoded by the full-length P protein mRNA.

Time course of synthesis. To substantiate this conclusion, it was important to rule out the possibility that the mRNA was being cleaved during translation. Toward that end, the placental ribonuclease inhibitor RNasin was included in all in vitro translation reactions. A direct experimental approach was also devised to measure the potential for cleavage of the RNA during translation.

It might be predicted that the amount of 7K protein synthesized relative to the amount of P protein would increase with time of incubation if the 7K protein was translated only after the mRNA was cleaved. Therefore, portions of a lysate programmed with in vitro poly(A)$^+$ VSV mRNA were removed at various times after the initiation of translation and snap frozen in dry ice-ethanol. The samples were thawed simultaneously, and the protein products were immunoprecipitated with polyclonal rabbit antiserum. After electrophoresis in a polyacrylamide gel containing SDS, the bands were cut out and counted.

In contrast to what may have been predicted, the ratio of 7K protein to P protein showed a general decrease with increasing time of incubation (Table 1). At 5 min there was approximately 0.35 mol of 7K protein per mol of P protein, whereas by 120 min the ratio had decreased to 0.13 mol/mol. This was totally inconsistent with synthesis of the 7K protein after cleavage of the mRNA. A decrease in this ratio may not have been totally unexpected if, for example, saturation of the mRNA with ribosomes translating downstream from the P protein initiation site (AUG_{11}) reduced the efficiency of initiation at the internal AUG codon.

Expression vector RNA. An RNA expression vector (pRH18) was constructed by subcloning the *Hinc*II A fragment of the pNS319 cDNA insert (9) into plasmid pSP64 (21). In this construction, nucleotides 382 to 814 (numbered from the 5′ end of the P protein mRNA) were inserted downstream from the bacteriophage SP6 RNA polymerase promoter. Transcription of the linearized template with the phage polymerase in vitro (21) yielded a large amount of pure plus sense P-protein-specific RNA.

The uncapped expression vector RNA was translated in a reticulocyte lysate under exactly the same conditions as those used to translate the normal capped viral messages.

TABLE 1. Ratio of proteins synthesized in vitro

Time of incubation (min)	Radioactivity (cpm) in protein: 7K	P	Ratio of 7K/P (cpm)	Ratio of 7K/P (mol)[a]
5	335	2,393	0.14	0.35
10	480	6,019	0.08	0.20
30	854	11,714	0.07	0.18
60	1,009	10,578	0.10	0.24
120	472	9,061	0.05	0.13

[a] Assumes that there are two methionines per 7K protein and five methionines per P protein (9).

Per microgram of template, the uncapped pRH18 RNA was approximately 50 to 60% as efficient as a mixture of mature in vitro VSV mRNAs in directing the incorporation of [^{35}S]methionine into acid-insoluble material. Analysis of the protein products of this reaction is shown in Fig. 5.

The pRH18 transcript directed the synthesis of three major products in vitro that were immunoprecipitated by polyclonal rabbit antiserum (Fig. 5, lane b), but neither alternative initiation nor termination codons were supplied by the vector in this plasmid. The products had molecular weights of approximately 13,000, 9,000, and 7,000. These sizes were consistent with the initiation of translation at three AUG codons (positions 512, 560, and 623 from the 5' end of the P protein mRNA, respectively) contained in the +1 reading frame, in phase with the P protein. Initiation at these AUGs would yield products with molecular weights of approximately 11,100, 9,100, and 6,900 (9; Rose, personal communication). The smallest of the expression vector products comigrated with the 7K protein encoded by the capped P protein mRNA (Fig. 5).

The synthesis of all three expression vector products was inhibited by hybrid arrest with the minus sense strand of the *Hpa*II B fragment of pNS173, but the minus sense strand of the *Hpa*II A fragment inhibited the synthesis of only the 9K and 13K products (data not shown).

Monoclonal antibodies against the P protein. Using the pRH18 expression vector products as antigens, I screened a library of monoclonal antibodies that was directed against the VSV P protein (provided by M. Williams and S. Emerson). Of the 11 antibodies tested, 4 precipitated all three expression vector products (representative results are shown in Fig. 5, lane c); a fifth antibody immunoprecipitated only the 9K and 13K products (results not shown). This showed that the expression vector products were translated in the same reading frame as the P protein and that some of the monoclonal antibodies recognized carboxy-proximal epitopes on the P protein. These results also demonstrated the utility of the pSP64 expression vector system for mapping the epitopes recognized by a collection of monoclonal antibodies.

Two of the four monoclonal antibodies identified as binding to carboxy-proximal epitopes were subsequently tested and found to immunoprecipitate both the P and 7K proteins that were translated from the mature capped in vitro VSV P protein mRNA (Fig. 5, lane d). This confirmed that the 7K protein was translated from the 3' end of the ORF encoding the P protein.

Intracellular proteins. Immunoprecipitation of ^{35}S-labeled proteins from a cytoplasmic extract of VSV-infected BHK cells with polyclonal rabbit antiserum detected the five known viral proteins and a small amount of a polypeptide with a molecular weight of about 7,000 (Fig. 6, lane e, arrow). A product of this size was not precipitated by the polyclonal antiserum from the lysate of an equal number of mock-infected BHK cells (Fig. 6, lane b). The inclusion of urea and three protease inhibitors made it very unlikely that this protein was generated by the artifactual cleavage of another viral product during immunoprecipitation from the cell lysate (see Materials and Methods for details).

Immunoprecipitation of the infected-cell lysate with a monoclonal antibody directed against a carboxy-proximal epitope of the P protein detected both the P protein and the small protein (Fig. 6, lane d, arrow). Some material which

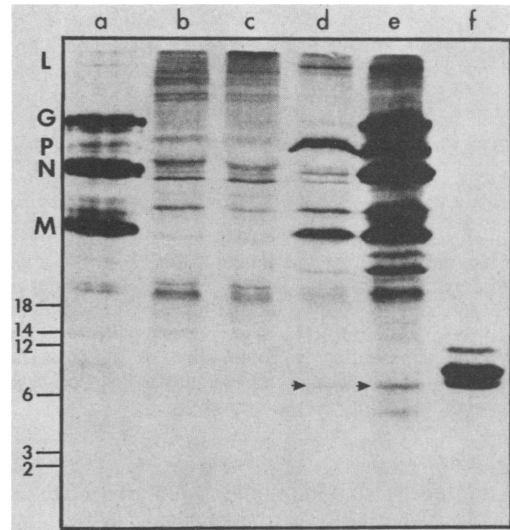

FIG. 6. Intracellular products. BHK cells infected with the Mudd/Summers isolate of VSV-Indiana or mock infected were labeled with [^{35}S]methionine between 2 and 5.5 h postinfection. Cytoplasmic extracts were prepared as outlined in Materials and Methods. Equal proportions of the extracts were immunoprecipitated and applied to the gel. Lanes: a, virion proteins, identified at left; b, mock-infected lysate, polyclonal antiserum; c, mock-infected lysate, monoclonal antiserum; d, infected-cell lysate, monoclonal antiserum; e, infected-cell lysate, polyclonal antiserum; f, pRH18 expression vector translation products, polyclonal antiserum. The locations of protein molecular weight standards are indicated as explained in the legend to Fig. 2.

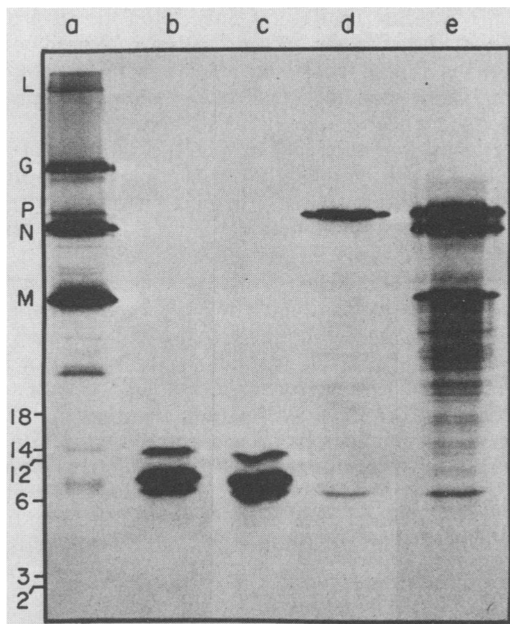

FIG. 5. Immunoprecipitation with monoclonal antibodies. Expression vector RNA (140 ng per reaction) or capped in vitro mRNA (80 ng per reaction) was translated under standard conditions. Lanes: a, virion proteins, identified at left; b, expression vector translation products immunoprecipitated with polyclonal rabbit antiserum; c, expression vector products precipitated with mouse monoclonal anti-P protein antibody; d, mRNA translation products immunoprecipitated with monoclonal antibody; e, mRNA products precipitated with polyclonal antiserum. The locations of protein molecular weight standards are indicated as explained in the legend to Fig. 2.

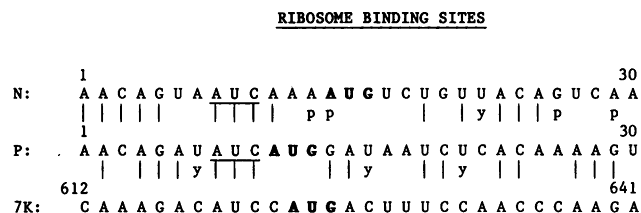

FIG. 7. Ribosome-binding sites. The sequences of the N protein and P protein message ribosome-binding sites (9, 30, 31) are aligned with the sequence surrounding AUG_{623} (9; Rose, personal communication) at the common 5'. . .AUC. . .3' triplet (underlined). Exact matches to the P protein mRNA sequence are indicated by vertical lines. p, Purine substitution; y, pyrimidine substitution. The AUG codons are highlighted. The numbers indicate nucleotide position from the 5' end of the plus sense strand.

comigrated with the viral M protein and a number of other minor bands were also precipitated. Immunoprecipitation by the monoclonal antibody in the presence of 0.72 M NaCl significantly reduced the levels of these nonspecific background bands but had only a minimal effect on precipitation of either the P protein or the small protein (data not shown). Regardless of the conditions used, the small protein was detected only in extracts of VSV-infected BHK cells and not in lysates of mock-infected cells.

An in vivo pulse-chase experiment indicated that the 7K protein turned over rapidly and did not accumulate in the cytoplasm of the infected cell (data not shown). This made it unlikely that the 7K protein was produced merely by the proteolytic cleavage of the P protein.

In well-resolved gels the small protein migrated slightly more rapidly than the 7K in vitro expression vector product (data not shown). This may have been indicative of an intracellular posttranslational modification of the 7K protein. Potential modifications included removal of the amino-terminal methionine residue in the infected-cell cytoplasm. That possibility makes accurate estimation of the in vivo ratio of 7K protein to P protein difficult at this time.The P protein message sequence from the San Juan isolate indicates that 5 and 2 methionines are encoded in the P and 7K proteins, respectively (9). Assuming that the sequence of the Mudd/Summers isolate used here predicts the same number of methionines, then a 2.5-fold difference in band intensity would be expected even if the two proteins were present in a 1:1 ratio and both amino-terminal methionines were conserved. The results shown in Fig. 6 suggest that the actual difference was larger. The observed intensity difference may have been as much as 1:10 (7K protein to P protein), and that would put the ratio of the two intracellular proteins into the same range as the ratio obtained in the in vitro experiments (Table 1, 120 min). Nevertheless, because the P protein was very abundant in the cytoplasm, there would still have been a significant number of copies of the 7K protein even at that ratio.

DISCUSSION

The results presented here demonstrate that in vitro and in vivo VSV P protein messages were translated in the reticulocyte lysate to yield two proteins. One of these comigrated with the authentic virion P protein. The other, a 7K protein, was encoded by the 3' one-third of the same ORF. A protein of approximately the same size and of similar antigenicity was also detected in a cytoplasmic extract of VSV-infected BHK cells. Synthesis of the 7K protein in vitro was unaffected by hybrid arrest conditions that blocked the 5' two-thirds of the message sequence; this rules out the possibility that the 7K polypeptide was produced merely by premature termination of translation or by proteolytic cleavage of the P protein. Blot analysis of the mRNAs detected only the normal P protein mRNA and two larger readthrough products. This indicates that the template for the 7K protein was probably the full-length P protein message. Significant degradation of the message during in vitro translation was unlikely because the ratio of 7K protein to P protein decreased with increasing time of incubation.

Indirect evidence against random cleavage of the P protein mRNA in the lysate was provided by the experiments with the pRH18 expression vector transcript, which can be viewed as a model randomly degraded message. This RNA initiated translation at all three AUG codons in ORF+1. Initiation at AUG_{560} (yielding the 9K product) was relatively efficient on the expression vector transcript, whereas initiation at this site on the capped P protein mRNA was not detected.

Evidence against site-specific cleavage of the mature mRNA in the lysate was provided by another uncapped expression vector transcript. This RNA contained nucleotides 33 to 814 of the P protein message sequence and also initiated translation at the three AUG codons in ORF+1 (data not shown). Therefore, if site-specific cleavage of the full-length P protein mRNA occurs, the specificity for the cleavage would have to reside within the first 32 nucleotides of the sequence.

A scanning model for the initiation of protein synthesis has been proposed by Kozak (15). A major premise of this model is that the 40S ribosomal subunits and associated factors bind at or near the capped 5' end of the mRNA and scan toward the interior until they encounter the 5'-proximal AUG codon. If this triplet is contained in a favored context, the ribosomes initiate translation. However, if the context is suboptimal, the ribosomes continue scanning until a more favorable sequence is encountered. Nevertheless, some ribosomes may initiate translation at the suboptimal AUG. This can lead to the synthesis of overlapping related proteins or, perhaps, to the synthesis of proteins in different reading frames. Both situations appear to occur during the translation of the Sendai virus P/C message (11).

The results presented here suggest that another process occurs during the translation of the VSV P protein message. Initiation at AUG_{623} was unaffected, or enhanced, by hybridization of the 5'-proximal portion of the sequence to an excess of cloned minus sense cDNA. According to the scanning model (15), the double-stranded region should have prevented the ribosomes from accessing that AUG codon. These results provide direct evidence for internal ribosome binding and initiation of translation at a site located hundreds of nucleotides downstream from the capped 5' end. This model predicts that the internal initiation of translation should be relatively insensitive to the presence of cap analogs which normally inhibit protein synthesis by competing for the cap binding protein (39). This hypothesis has been tested and appears to be correct (unpublished observations).

The ribosomes must be selective in their choice of an internal start codon because only AUG_{623} is used to initiate translation on the capped P protein mRNA even though it is in a suboptimal context according to the scanning model (15). A comparison of the ribosome-binding sites on the N and P protein messages (9, 30, 31) with the sequence surrounding AUG_{623} in the P protein mRNA is shown in Fig. 7. The three sequences have been aligned relative to the common 5'. . .AUC. . .3'. This triplet is found in all VSV

ribosome-binding sites with the exception of that on the G protein- mRNA (31) and could conceivably hybridize to the 3'. . .UAG. . .5' that is located near the 3' end of the 18S ribosomal RNA (34).

The sequence surrounding AUG_{623} clearly resembles the major P protein message ribosome-binding site. The two sequences are identical in 16 out of 30 positions (53%), and there are pyrimidines in the same place at three other sites (10%). In comparison, the N protein and major P protein message ribosome-binding sites share 14 identical positions (47%), and there are five places (17%) at which both have either a purine or pyrimidine.

Two groups have independently isolated mutants which they assigned to a sixth VSV-Indiana genetic complementation group (8, 28). The published data are consistent with the conclusion that the mutation in at least one of these is actually within the P protein cistron (4). Those are precisely the results to be expected if a second protein is encoded by the P protein mRNA. The myriad of phenotypes assigned to complementation group II (P protein) mutants may also be equally indicative of multiple proteins as well as of multiple functions for the P protein. The role of the 7K protein during the VSV growth cycle is under investigation.

Small polypeptides encoded in the same ORF as the P protein appear to be common among nonsegmented negative-strand viruses. Newcastle disease virus (5), mumps virus (35), and simian virus 5 (27) all translate smaller nonstructural proteins in phase with the P protein. Collins et al. (5) reported that the two NS proteins of Newcastle disease virus could be labeled in vitro with formyl-[^{35}S]methionine. On the basis of that observation they tentatively concluded that the NS proteins represent portions of the amino terminus of the Newcastle disease virus P protein. By analogy, Simpson et al. (35) made a similar suggestion for the NS proteins of mumps virus. In view of the data presented here, the possibility that these proteins are produced by an internal initiation of translation must now be considered. The implications of this phenomenon for translation of the mRNAs encoded by other viruses, including picornaviruses, coronaviruses, and flaviviruses, should also be investigated.

Comparison of the sequences of the P protein messages from the Indiana and New Jersey serotypes of VSV by Gill and Banerjee (10) indicates that the predicted carboxy termini are the most highly conserved regions of the two proteins. They have remarkable homology in region III (10), and this suggests that some important function may be encoded therein. If proximity to a 5'. . .AUC. . .3' triplet is significant for VSV translation (see above), then two AUG codons near the 3' end of the VSV-New Jersey P protein mRNA have the potential to serve as internal initiation sites. One of these is immediately adjacent to two such triplets and is located within the highly conserved region III (10). The sequence surrounding this AUG codon is identical to the first 30 nucleotides of the mRNA (presumably containing the 5' ribosome-binding site) in 12 positions. Internal initiation at this AUG codon predicts a very small product containing only 18 amino acid residues. The other AUG codon is located 24 nucleotides downstream from an AUC triplet, and its sequence context is not very similar to the 5' end of the mRNA; initiation at this site predicts a protein containing 49 amino acid residues. The potential use of either of these codons remains to be determined.

Because of the existence of the 7K protein, I believe that it is now appropriate to revise the nomenclature used for the VSV P protein. This protein has been designated NS because it was originally thought to be a nonstructural product. Work from numerous laboratories has since shown it to be an essential virion component despite its relatively low abundance. Therefore, in keeping with the nomenclature used for other negative-strand viruses, this cistron, its mRNA, and the protein should be redesignated P.

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