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Translation Initiation at Alternate In-Frame AUG Codons in the Rabies Virus Phosphoprotein mRNA Is Mediated by a Ribosomal Leaky Scanning Mechanism

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The phosphoprotein of rabies virus is a 297-amino-acid polypeptide encoded by the longest open reading frame of the P gene. Immunoprecipitation experiments using a monoclonal antiserum directed against the P protein detected the P protein and at least four additional shorter products in infected cells, cells transfected with a plasmid encoding the wild-type P protein, and purified virus (CVS strain). By means of deletion analyses, these proteins were shown to be translated from secondary downstream in-frame AUG initiation codons. Immunofluorescence experiments indicated that all these P products were found in the cytoplasm of transfected cells; however, the proteins initiated from the third, fourth, and fifth AUG codons were found mostly in the nucleus. Changes in the 5′-terminal region of the P mRNA (including site-specific mutations, deletions, and insertions) demonstrated that a leaky scanning mechanism is responsible for translation initiation of the P gene at several sites.

Rabies virus is a member of the rhabdovirus family. Rhabdoviruses have a single-stranded RNA genome of a negative polarity which acts as a template for transcription that produces a positive-strand leader RNA and five capped, methylated, polyadenylated mRNAs (1, 33). These mRNAs are usually considered monocistronic because they each encode a single translation product: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), and the RNA polymerase (L). However, studies of vesicular stomatitis virus (VSV), the prototypical rhabdovirus, have shown that the mRNA encoding phosphoprotein P directs the synthesis of more than one protein and is thus polycistronic. These proteins may be encoded by the same open reading frame (ORF) (14) or by overlapping ORFs (29). It is interesting that for most single-stranded negative RNA viruses (paramyxoviruses and rhabdoviruses), the P gene has been shown to encode multiple proteins.

Two mechanisms for generating more than one protein from a single mRNA have been described for several negative-strand RNA viruses, mostly for paramyxoviruses (6, 7). One possibility is through leaky scanning, as proposed by Kozak (17). In this model, ribosomes bind at the capped 5′ end of the mRNA and scan downstream to the first AUG codon. Failure to initiate translation at this codon would result in continued scanning downstream from the next available AUG; a second failure allows recognition at a third AUG. The other possibility is a cap-independent mechanism with internal ribosome initiation as reported for the translation of the picornavirus polyprotein and Sendai virus proteins (6, 23).

The VSV P protein is a multifunctional regulatory protein which plays a key role in viral transcription and replication; it is associated with the L protein in the polymerase complex and forms complexes with both soluble and genome-associated N proteins (9, 19, 24). Moreover, the P protein has different phosphorylation states, thought to bind with different affinities to the ribonucleoprotein template and to exhibit different transcription activities (2). The structural similarities between rabies virus and VSV suggest that rabies virus phosphoprotein has the same properties. Two domains of the rabies virus P protein are involved in vitro and in vivo in the interaction with N protein (4, 11) as previously reported for VSV (30). In addition, at least two phosphorylated forms of P are also found in mature rabies virus (32).

In this paper, we report direct evidence for four additional proteins derived from the P gene of rabies virus (CVS strain) in infected cells, in cells transfected with a plasmid encoding the wild-type P protein, and in purified virus. Deletion analyses show that these proteins are initiated from secondary downstream in-frame AUG initiation codons. The 5′-terminal region of the P mRNA was modified by site-specific mutations, deletions, and insertions to study the mechanism of translation initiation of the P gene. The results show that translation is initiated at secondary downstream AUG codons by a leaky scanning mechanism. Immunofluorescence experiments indicated that the proteins initiated from the third, fourth, and fifth AUG codons were found mostly in the nucleus, whereas the wild-type P protein and the proteins initiated from the second AUG were present in the cytoplasm.

MATERIALS AND METHODS

Cells and viruses. BSR cells, a clone of BHK 21 (baby hamster kidney) cells were grown in Eagle minimal essential medium supplemented with 10% calf serum. NG108-15 mouse neuroblastoma-rat glioma hybrid cells were cultured as described previously (15).

The CVS strain of rabies virus was cultivated and purified as previously described (13). Recombinant vaccinia virus (VTF7-3) containing the T7 RNA polymerase gene has been described previously (12) and was kindly provided by B. Moss, National Institutes of Health, Bethesda, Md.

Plasmid constructions. DNA manipulations were carried out by the methods described by Sambrook et al. (26).

(i) Plasmids pMC.P and pBS.P. Both pMC.P and pBS.P contain the wild-type P gene; pMC.P has been described previously (4); pBS.P was obtained by inserting the *Xba*I fragment corresponding to the P gene from pMC.P into the *Xba*I site of the Bluescript SK vector (Stratagene).

(ii) Plasmids pPA2, pPA3, pPA4, and pPA5. pPA2, pPA3, pPA4, and pPA5 differed from pMC.P by deletions at the 5′ terminus of the P gene of 77, 178, 225, and 265 bp, respectively. These deletions were introduced by PCR amplification of the wild-type P gene, using the synthetic oligonucleotides, Pa2, Pa3, Pa4, and

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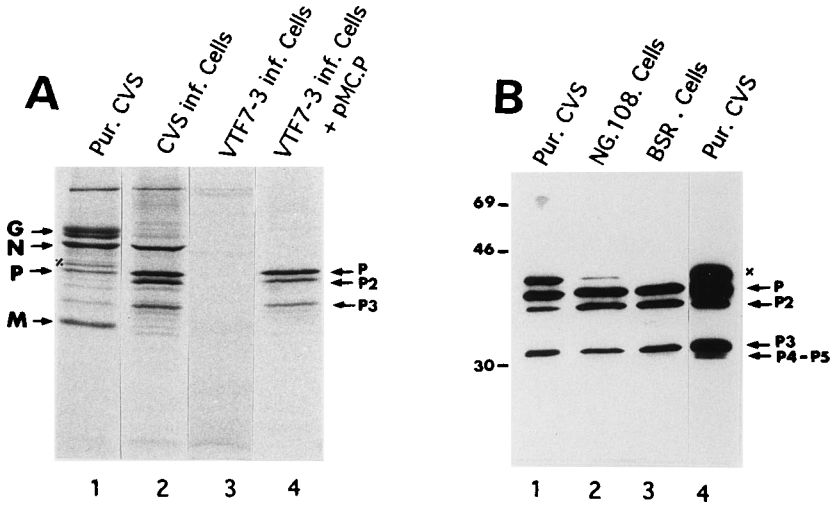

FIG. 1. Detection of truncated forms of P protein in infected and transfected cells and in purified virus. (A) Immunoprecipitation. BSR cells were infected (inf.) with rabies virus (lane 2) or with the VTF7-3 recombinant vaccinia virus (lane 3). VTF7-3-infected cells were transfected with plasmid pMC.P encoding the wild-type P protein (lane 4). ^{35}S-labeled proteins were immunoprecipitated with the monoclonal A17 anti-P antibody (lanes 2, 3, and 4). Immunoprecipitates were analyzed by SDS-PAGE (12% polyacrylamide). [^{35}S]methionine-labeled proteins from purified (pur.) virus are included for comparison (lane 1). (B) Immunoblotting. Proteins from rabies virus-infected cells (NG108-15 [lane 2] and BSR [lane 3]) and from purified virus (lanes 1 and 4) were analyzed by Western blotting. The blot was immunostained with the A17 anti-P antibody and visualized by the ECL system (Amersham). A longer exposure of the blot containing the purified virus is included in lane 4. The positions of molecular standard markers (in kilodaltons) are shown on the left (^{14}C methylated; Amersham). The hyperphosphorylated form of P is indicated by an asterisk.

Pa5, respectively, and the reverse primer PB. Pa2 (GCCTCTAGACGATCTTG AG**ATG**GC) contains the sequence corresponding to nucleotides 77 to 91 of the P mRNA, thus including the in-frame ATG2 (in boldface letters). Pa3 (GC CTCTAGACTGAGGAC**ATG**AAGC) contains the sequence corresponding to nucleotides 178 to 192 of the P mRNA, including the in-frame ATG3 (in boldface letters). Pa4 (GCCTCTAGACTTGGTGAG**ATG**GTT) contains the sequence corresponding to nucleotides 225 to 239 of the P mRNA, and thus includes the in-frame ATG4 (in boldface letters). Pa5 (GCCTCTAGAAGG ACTTTCAG**ATG**G) contains the sequence corresponding to nucleotides 265 to 279 of the P mRNA, including the in-frame ATG5 (in boldface letters). Every oligonucleotide contains an *Xba*I cloning site (underlined) plus a GCC codon at its 5′ end. PB [GCCTCTAGA$(dT)_{12}$CAT] has been described previously (4). The amplified double-stranded cDNAs corresponding to deleted P genes were digested with *Xba*I and were inserted into the corresponding cloning sites of the expression vector CDM8 (12).

(iii) Plasmid pMC.PH. pMC.PH was constructed by inserting a head-to-tail repeat of the 39-bp *Pst*I-*Eco*RI fragment of the multiple cloning site sequence of pUC18 (Boehringer) into the *Pst*I site of pBS.P.

(iv) Plasmids pPK1, pPK2, pPK3, and pPK4. pPK1, pPK2, pPK3, and pPK4 were constructed by site-directed mutagenesis of the plasmid pBS.P by PCR using the oligonucleotides Pk1, Pk2, Pk3, and PB. Pk1 (GCCCTGCAGCCAC C**ATG**AGCAAGATCTTTG) contains the sequence corresponding to nucleotides 30 to 45 of the P mRNA including the in-frame ATG1 (in boldface letters) in a context considered favorable for initiation, as described by Kozak except that the A immediately downstream of the initiation codon is unchanged (17); Pk2 (GCCCTGCAGCCACC**ATG**GGCAAGATCTTTG) contains the same sequence as Pk1 except that the A following the initiation codon is replaced by G and the context is thus optimal for initiation (17). Pk3 (GCCCTGCAGACCA TCCCATCT**ATG**TGCAAGATCTTTG) contains the sequence corresponding to nucleotides 18 to 45 of the P mRNA, including the in-frame ATG1 in a context considered unfavorable for initiation (in boldface letters). Pk4 (GCCCTGCAG ACCATCCCAAAT**CCG**AGCAAGATCTTTG) contains the sequence corresponding to nucleotides 18 to 45 of the P mRNA in which ATG1 was replaced by CCG. Every oligonucleotide contained an *Xba*I cloning site (underlined) plus a GCC codon at its 5′ end. The amplified double-stranded cDNAs were digested with *Pst*I and *Xba*I and were inserted into the corresponding cloning sites of the Bluescript vector (Stratagene).

(v) Plasmid pPΔc120. pPΔc120 has been described previously (4).

DNA transfection. Proteins were transiently expressed by using a T7 vaccinia virus expression system according to the method of Fuerst et al. (12). Briefly, BSR cells were grown in 3.5-cm-diameter dishes to about 80% confluency and were infected with VTF7-3 at a multiplicity of infection of 5 PFU per cell. After 1 h of adsorption, the cells were transfected with 5 μg of supercoiled plasmid DNA by the calcium phosphate coprecipitation procedure (21).

Radiolabeling and immunoprecipitation of viral proteins. Twenty-four hours after infection or after transfection, proteins were labeled with 50 μCi of [^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham) per ml or with 80 μCi of $^{32}P_i$ (10 mCi/ml; Amersham) per ml for 4 h. Cells were harvested by scraping and were lysed in 50 mM Tris-HCl (pH 7.5)–0.5% Nonidet P-40 in the presence of a mixture of protease inhibitors (CLAPA: 1 μg each of chymostatin, leupeptin, antipain, and pepstatin per ml and 8 μg of aprotinin per ml). Nuclei were eliminated from the lysate by centrifugation at 10,000 × *g* for 5 min at 4°C. The cytoplasmic fractions were incubated overnight at 4°C with the specific murine monoclonal anti-P antibody whose epitope has been mapped between amino acids 69 and 177 of the P protein (4). Immune complexes were precipitated by adding protein A-Sepharose (Sigma), washed three times in the lysis buffer and analyzed by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

Immunoblotting. Proteins were analyzed by Western immunoblotting and revealed by peroxidase activity detection with the light-based ECL system as described by the manufacturer (Amersham).

Indirect immunofluorescence staining. Twenty-four hours after transfection and infection, transfected or infected cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.5, for 10 min and permeabilized for 5 min with 0.05% Triton X-100 in PBS. Viral P protein was stained with the mouse monoclonal A17 anti-P antibody and the corresponding goat anti-mouse, fluorescein-conjugated antibody (Cappel).

RESULTS

Presence of small P gene-encoded proteins in cells expressing wild-type P protein and in rabies virus-infected cells. Cells were infected with rabies virus (CVS strain) and labeled with ^{35}S, and cell extracts were immunoprecipitated with the A17 monoclonal anti-P antibody which recognizes an epitope located between amino acids 69 and 177 of the P protein (4). The immunoprecipitates were analyzed by SDS-PAGE (Fig. 1). As previously reported (4), the anti-P antibody immunoprecipitated the P protein and the P-N complexes (Fig. 1A, lane 2). In addition, two smaller protein bands, P2 and P3, were detected (Fig. 1, lane 2). A P4-P5 doublet was also detected after loading of excess immunoprecipitate or after overexposure of the autoradiogram. To determine whether these forms were present in cells expressing the wild-type P protein, cells were infected with VTF7-3 recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (12) and were then transfected with plasmid containing the wild-type P gene (pMC.P) under the control of the T7 RNA polymerase promoter. The P protein, P2, and P3 were present in the transfected cell extracts

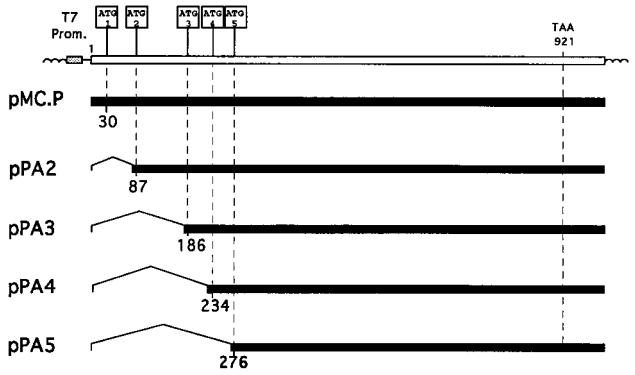

FIG. 2. Schematic representation of plasmids encoding N-terminally truncated P proteins. Plasmids pPA2, pPA3, pPA4, and pPA5 were obtained from pMC.P by deletions including the in-frame ATG at the 5′ end of the P gene as described in Materials and Methods. Wild-type or 5′-truncated P genes (solid bars) were under the control of the T7 promoter (prom.). The thin angled lines indicate deleted regions. Nucleotide positions are indicated by the numbers at the left of the bars.

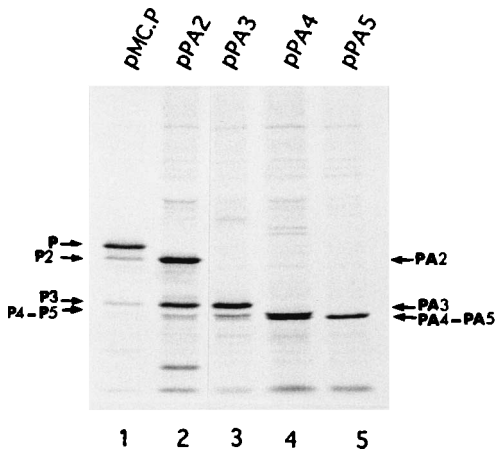

FIG. 3. SDS-PAGE analysis of immunoprecipitated proteins from cells transfected with plasmids encoding N-terminally truncated P proteins. BSR cells were infected with VTF7-3 and were then transfected with plasmid pMC.P encoding wild-type P protein (lane 1) or with plasmids encoding truncated P proteins pPA2 (lane 2), pPA3 (lane 3), pPA4 (lane 4), and pPA5 (lane 5). [^{35}S]methionine-labeled proteins were immunoprecipitated from cell extracts with A17 anti-P antibody (lanes 1 to 5).

immunoprecipitated by A17 (Fig. 1A, lane 4). None of these proteins were present in uninfected cell extracts (Fig. 1A, lane 3). To investigate whether these forms were present in other rabies virus-infected cells and in purified virus, the pattern of the proteins present in CVS-infected cells was compared with that of proteins detected in the virus by immunoblotting with the A17 antibody. As shown in Fig. 1B, lane 2, P2 and P3 were also detected in infected NG108-15 neuroblastoma cells. Two phosphorylated forms were present in the virus (32): the upper band corresponds to the hyperphosphorylated form (Fig. 1B, asterisk), and the lower corresponds to the hypophosphorylated form (32). The P protein found in infected cells comigrated with the hypophosphorylated form from the virus (Fig. 1B, lanes 1 to 4) as previously mentioned (4). However, the P2 and P3 proteins were also present in the mature virus (Fig. 1B, lanes 2 and 4). Although this experiment did not yield quantitative results, P3 appeared to be more abundant than P2 in the virus. After longer exposures, the P4-P5 doublet could be seen (Fig. 1B, lane 4). These results suggest that these products (P2, P3, P4, and P5) are immunologically related, virus-specific, and encoded by the P gene. They could not have been the result of proteolytic cleavage, because they were present when the cell extracts were obtained in the presence of a protease inhibitor cocktail (data not shown). In addition, a Northern (RNA) blot analysis indicated that these P forms were not translated from shorter forms of P mRNA (data not shown). Thus, the template for short P proteins was most likely the full-length P messenger and these proteins were presumably initiated at secondary in-frame AUG codons.

Short P proteins are translated from alternative in-frame AUG codons in P mRNA. Sequence analysis of the P gene of strain CVS suggests that the coding region for the phosphoprotein starts from the first AUG codon, which is located 30 nucleotides from the 5′ end of the mRNA. This would yield a 297-amino-acid protein (25). The same ORF contains four downstream AUG codons at positions 87, 186, 234, and 276 of the P gene sequence. The estimated molecular weights of P2, P3, P4, and P5 were consistent with the initiation of their translation at these four AUG codons.

To investigate the origin of these P forms, mutated P genes were obtained by deletion of 77, 178, 225, or 265 nucleotides at the 5′ terminus of the P mRNA such that one to four AUG codons were deleted (Fig. 2). It is worth noting that the sequence initiation context preceding each AUG was conserved in the mutated P genes (Fig. 2). To analyze translation initiation at these internal AUG codons, cells were transfected with plasmids encoding the N-terminally truncated P proteins and ^{35}S-labeled proteins were immunoprecipitated with the A17 antibody. No protein band corresponding to the wild-type P protein was produced from the 5′-terminal 77-nucleotide deletion (pPA2) (Fig. 3, lane 2). However, a protein named PA2 which comigrated with P2 was present in large amounts (Fig. 3, lanes 1 and 2). Three other polypeptides migrating like P3 (named PA3) and like the P4-P5 doublet (named PA4-PA5) were immunoprecipitated (Fig. 3, lane 2). A larger deletion of 178 nucleotides including ATG2 and preceding ATG3 (pPA3) resulted, as expected, in the loss of PA2 and in a concomitant increase in the amounts of PA3 comigrating with P3 (Fig. 3, lane 3). In addition, the amounts of the polypeptides PA4 and PA5 were increased (Fig. 3, lane 3). The extension of the deletion to 225 nucleotides preceding ATG4 (pPA4) prevented the synthesis of PA3 and led to increased amounts of PA4 and PA5 (Fig. 3, lane 4). When the four ATG codons were deleted (265 nucleotides) (pPA5), we obtained a maximal amount of PA5 and no synthesis of PA2, PA3, and PA4 (Fig. 3, lane 5). These results indicate that P products P2, P3, P4, and P5 correspond to truncated proteins PA2, PA3, PA4, and PA5, respectively, and thus that they are initiated from secondary, downstream, in-frame AUG initiation codons. In addition, these polypeptides were phosphorylated in vivo in infected and in transfected cells (data not shown), suggesting that potential sites for phosphorylation in CVS P protein are not removed or are only partially removed by deletion.

Study of the translation mechanism of P mRNA. There are two possible mechanisms for P gene translation. The first is leaky scanning as proposed by Kozak (17). In this model, ribosomes scan from the 5′ end of the mRNA until they reach the first AUG initiation codon and then bypass the 5′ proximal AUG and initiate protein synthesis at the second AUG (17). The second is internal ribosome initiation, which is a cap-independent mechanism, as described for the picornaviruses (23).

A direct approach to determining which mechanism applies

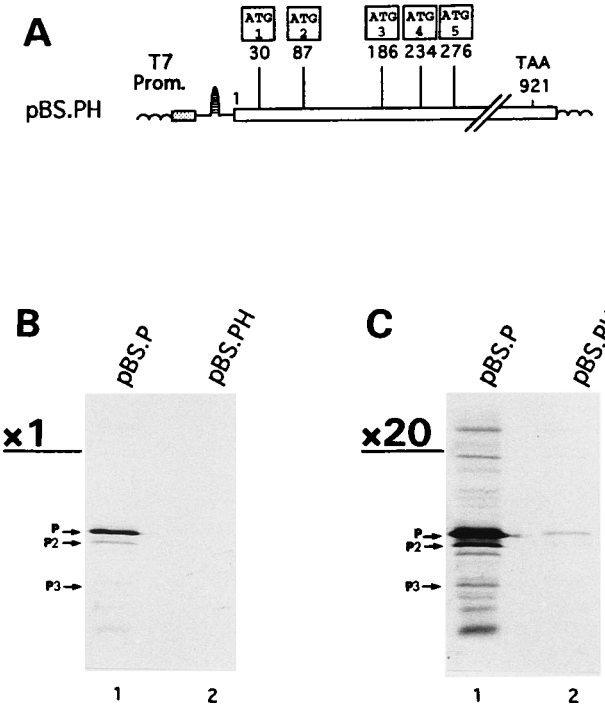

FIG. 4. Inhibition of P mRNA translation by introduction of a hairpin at its 5′ extremity. (A) Schematic representation of pBS.PH. Plasmid pBS.PH is identical to pBS.P except that it contains an inverted tandem repeat of 39 bp inserted in the *Pst*I site downstream from the T7 promoter (prom.). (B) [^{35}S]methionine-labeled immunoprecipitated proteins from pBS.P (lane 1)- and pBS.PH (lane 2)-transfected cells analyzed by SDS-PAGE. (C) An exposure of the same proteins 20 times longer than that in panel B.

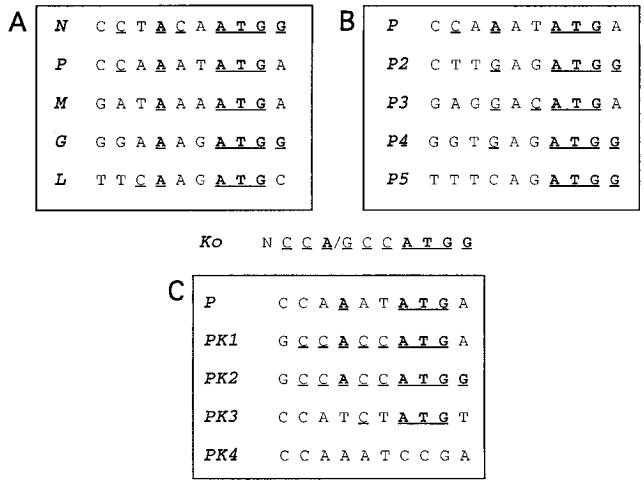

FIG. 5. Nucleotide sequences of initiation regions of the rabies virus L, N, G, P, and M2 proteins (A); P, P1, P2, P3, and P4 proteins (B); and site-directed mutants pPK1, pPK2, pPK3, and pPK4 (C). The central sequence (*Ko*) represents the optimal context as described by Kozak (17). Note that all the sequences of the initiation context except that of the L gene correspond to the CVS strain. The sequence of the L gene (CVS strain) has not been determined, and we have reported the seuqences of SAD-B19 (5) and strain PV (31), which are identical.

is to introduce a stable hairpin structure at the 5′ end of the noncoding region. Hairpins have been shown to prevent scanning ribosomes from initiating translation at downstream AUG initiation codons (23). Thus, an inverted tandem repeat (a 39-bp *Eco*RI-*Pst*I fragment from pUC18) was inserted into the *Pst*I site of pBS.P encoding the wild-type P protein to produce plasmid pBS.PH (Fig. 4A). Northern blot analysis of mRNA confirmed that the hairpin did not modify the transcription efficiency at the T7 promoter (data not shown). Cells were transfected with the plasmid pBS.PH. No P gene product was detected at a standard autoradiography exposure (Fig. 4B). Only a weak band was visible after overexposure (20×) (Fig. 4C). Thus, the hairpin structure inhibited the translation initiation of P, P2, P3, P4, and P5. This result strongly suggests that the P protein is initiated by ribosomes scanning from the 5′ end of the P mRNA and that the truncated P proteins are translated by a leaky scanning mechanism from the 5′ end of the P messenger.

Effect on P translation of sequence context alteration around the proximal AUG. Kozak's rules for ribosomal scanning state that leaky scanning through an upstream AUG can occur only when this AUG is in a weak context. However, AUG1 is considered to be in a quite favorable context according to the Kozak consensus sequence (A in position −3) (Fig. 5C) (17). Moreover, this context appears as favorable as the context of the proximal AUG of other rabies virus genes which all have an A in position −3 and a G or an A in position +4 (Fig. 5A). To examine the involvement of the nucleotide sequence context of the first initiation codon of P mRNA, we constructed site-directed mutations in the initiation region, using oligonucleotides (Fig. 5C). The nucleotide sequence of the first AUG from the wild-type 5′ end (CAAAT**ATG**A) (pBS.P) was changed to a context predicted to be more favorable (C**C**AC**C****ATG**A) (pPK1) or optimal (C**C**AC**C****ATG**G) (pPK2) or highly unfavorable (CA**T**C**T****ATG**T) (pPK3) according to Kozak (17). Other mutations in which the initiation codon was changed to CCG (AAAT**CCG**A) were made (Fig. 5C). In cells transfected with pPK1, the relative amounts of the P protein and the truncated P forms were unchanged (data not shown). However, a G rather than an A in position +4 (pPK2) increased P translation and consequently prevented P2 and P3 protein synthesis (Fig. 6, lane 3). In contrast, when the ATG context was poor (pPK3), the expression of P was reduced and P2 and P3 protein synthesis was increased (Fig. 6A, lane 2). The construct in which the P initiation codon was eliminated (mutation ATG/CCG [pPK4]) produced large amounts of P2

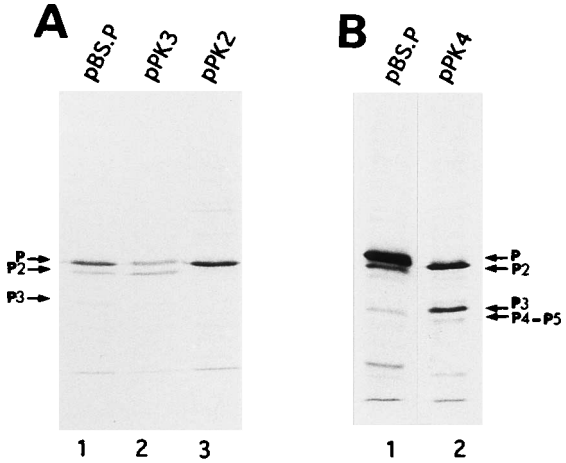

FIG. 6. Effect of site-directed mutation on P translation. (A) Cells transfected with mutants pBS.P (lane 1), pPK2 (lane 3), and pPK3 (lane 2). (B) Cells were also transfected with pPK4 (lane 2). These plasmids are described in Fig. 5. [^{35}S]methionine-labeled immunoprecipitated proteins were analyzed by SDS-PAGE.

and P3 and no P (Fig. 6B, lane 2). These results confirm that the P gene is initiated by a leaky scanning mechanism. Moreover, they show that the initiation context of the proximal AUG is important for P gene translation and that the introduction of the consensus Kozak sequence (G in position +4) totally prevents downstream scanning. Comparison of the nucleotide sequences around the four downstream AUG codons indicates that the initiation contexts are quite favorable for AUG2, AUG3, and AUG4 (G in position −3 and A or G in position +4) but are weaker for AUG5 (C in position −3) (Fig. 5B). These data are consistent with the leaky scanning mechanism.

Intracellular localization of P forms in transfected cells. Immunostaining experiments showed that cells transfected with plasmid encoding wild-type P protein (pMC.P) exhibited diffuse staining throughout the entire cell, with little nuclear fluorescence (Fig. 7A). We studied the intracellular localization of the N-terminally truncated P proteins in transfected cells. Staining of PA2 produced in BSR cells was mostly localized in the cytoplasm, with weak staining in the nucleus, as described for the wild-type P protein (data not shown). The truncated proteins PA3, PA4, and PA5 were found mainly in nuclei (Fig. 7B; data not shown for PA4 and PA5). This result indicates that a truncation of the N-terminal 52 amino acids of P protein is sufficient to allow entry into the nucleus. The migration of these small proteins to the nucleus may be the result of passive diffusion: however, truncation of the C-terminal 120 amino acids of P protein (PΔc120) did not result in migration to the nucleus (Fig. 7C) (4).

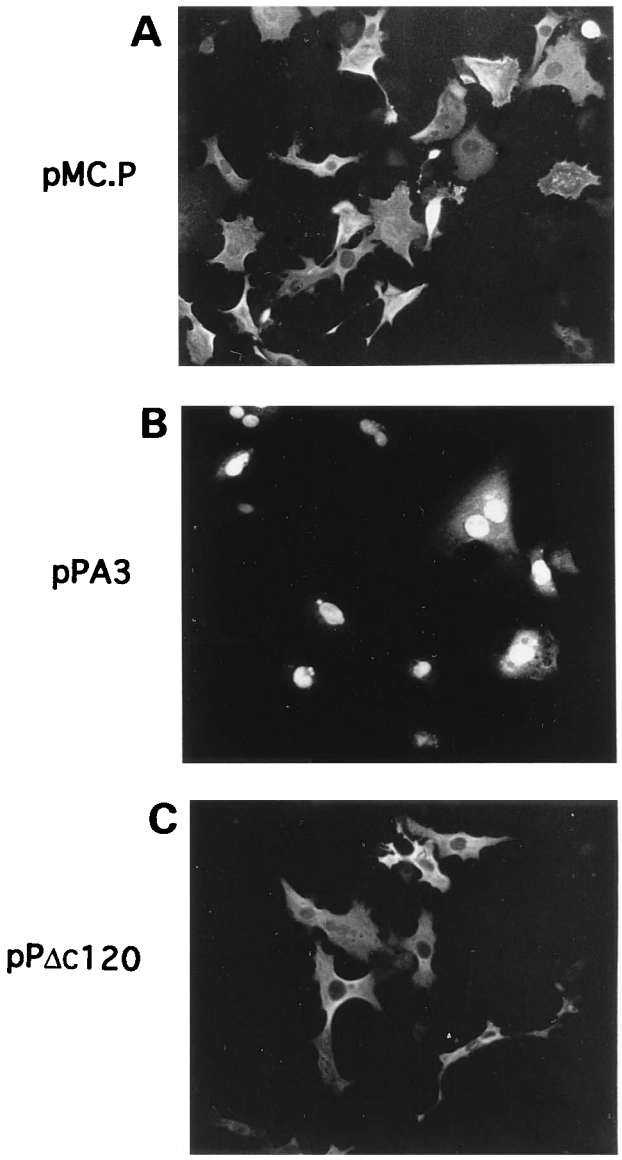

FIG. 7. Immunostaining of truncated P proteins expressed in transfected cells. Cells were transfected as described in Materials and Methods with plasmids pMC.P (A), pPA3 (B), and pPΔc120 (C). The distributions of P proteins in the cells were analyzed by indirect immunofluorescence with A17 anti-P antibody followed by incubation with goat anti-mouse immunoglobulin G coupled to fluorescein.

DISCUSSION

In this report, we demonstrated that five P gene products (P, P2, P3, P4, and P5) recognized by a monoclonal anti-P antibody are present in different rabies virus-infected cells (BSR cells and NG108-15 neuroblastoma cells) and in transfected cells expressing the wild-type P gene. These polypeptides are also present in purified virus. P2, P3, P4, and P5 are encoded by a single ORF and may be produced from AUG codons located at positions 87, 186, 234, and 276 of the P gene, respectively. Sequence comparison with the lyssavirus P gene indicates that AUG2 and AUG3 are conserved, whereas AUG4 and AUG5 are not (25). This is in agreement with the fact that P2 and P3 (initiated from ATG2 and ATG3, respectively) were present in larger amounts than P4 and P5 in infected cells and in virus preparation. However, we cannot exclude the possibility that these truncated P proteins are initiated from non-AUG codons, even if these events are very rare. It is interesting that the P genes of rhabdoviruses and paramyxoviruses are the only viral genes shown to encode more than one protein. For VSV, Herman has shown that an additional small protein (7 kDa) is synthesized from P mRNA (14) and recently, two proteins derived from the P gene second ORF have been reported (29). The P mRNAs of Sendai virus, Newcastle disease virus, simian virus 5, and measles virus also have been described as encoding multiple proteins (3, 6, 7, 20, 22).

We investigated the mechanism by which translation of these polypeptides is initiated. An RNA hairpin inserted close to the cap which blocks the ribosome entry at the 5′ end of the P gene inhibited the translation of all the P products. This suggested that the translation of the P gene is probably initiated by a leaky scanning mechanism rather than by internal ribosome entry. A major premise of this model is that the ribosomes bind near the capped 5′ proximal AUG. If this codon is in a favorable context, the ribosomes will initiate translation. If the context is unfavorable, some ribosomes continue scanning and thus find downstream AUG codons (16, 17). However, in the P mRNA, the start initiation codon for the P protein (located at position 30) is considered to be in a favorable context (25). To clarify this apparent discrepancy, directed mutagenesis was performed to modify the P gene initiation context. The results are in agreement with Kozak's rules and show that the introduction of the optimal Kozak consensus sequence with a G in position +4 prevents scanning and initiation at downstream AUGs. It should be noted that for the other viral N and G genes which have not been shown to encode multiple proteins, a G is present in position +4 (Fig. 5). It would be interesting to investigate the existence of shorter M or L products translated from the M or L genes

(CVS strain) which contain, respectively, an A or a C in position +4.

Recent studies have demonstrated that leaky scanning occurs on several polycistronic viral mRNAs, including P of hepatitis B virus (10, 18), VPu and Env of human immunodeficiency virus (27), and LP1 and VP1 in simian virus 40 (28). For Sendai virus, two mechanisms, leaky scanning and internal ribosome initiation, have been described for the translation of P/C mRNA (6, 7). For VSV, the 7-kDa protein has been shown to be synthesized from P mRNA by internal ribosome initiation (14) but the mechanism of initiation of the two proteins derived from the P gene second ORF of VSV has not been studied (29). Our data indicate that the rabies virus P mRNA can be considered polycistronic, as described for P mRNA of most paramyxoviruses and VSV. We did not investigate the existence of a potential second ORF in the CVS P gene as reported for VSV (29). However, close to the in-frame ATG3 (at base 208), there is an ATG in a quite favorable context which could lead to the synthesis of a short protein.

Immunofluorescence staining experiments performed on transfected cells indicate that P3, P4, and P5 initiated from, respectively, the third, fourth, and fifth AUG codons were found mostly in the nucleus, whereas the wild-type P protein and P2 were mainly cytoplasmic. Thus, a truncation of at least the N-terminal 52 amino acids of P protein appears to result in the ability to enter the nucleus. Two possible explanations are that the N-terminal deletions remove a cytoplasmic retention signal or on the contrary expose a nuclear localization signal. However, no such sequences were found by sequence searches of the P gene. Further experiments are thus required to determine if this migration is the result of a passive diffusion or an active process. Either way, this nuclear localization may have biologic effects on virus multiplication. At present, only speculation is possible about the function(s) of these small proteins. P2 and P3 are present in large amounts in purified virions. This suggests that they are involved in one of the steps of viral multiplication in infected cells, perhaps in transcription or replication as reported for the wild-type P protein. Indeed, these P forms contain the two binding sites recently shown to be required for association with nucleoprotein N (4). However, of the various proteins expressed from the P gene of paramyxovirus, only P is essential for RNA synthesis. All the other P-related proteins shown to affect RNA synthesis do so negatively (8). In further experiments, we plan to investigate the potential role of these additional P proteins.

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