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Characterization of the Internal Initiation of Translation on the Vesicular Stomatitis Virus Phosphoprotein mRNA

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ABSTRACT: Internal initiation of translation on the vesicular stomatitis virus (VSV) phosphoprotein (P) mRNA leads to the synthesis of a second protein [Herman, R. C. (1986) *J. Virol. 58*, 797–804]. Characterization of this phenomenon shows that initiation at the 5′-proximal and internal AUG codons has different optima for mono- and divalent cations in the reticulocyte lysate. Whereas 5′ initiation is stimulated by increasing concentration of K^+ over the endogenous level, internal initiation is inhibited. Internal initiation is much less sensitive to the effects of the cap analogue 7mGpppG in both the reticulocyte lysate and the wheat-germ extract under conditions that reduce 5′-proximal initiation to only about 4–5% of the control level. These results imply that 5′-proximal and internal initiations are distinct biochemical processes.

I recently reported that the 814-nucleotide vesicular stomatitis virus (VSV)[1] phosphoprotein (P) mRNA encodes a second product (M_r 7000; 7K protein) within the 3′ third of the same open reading frame (ORF) encoding the phosphoprotein (Herman, 1986). Translation of this small protein from the capped full-length P mRNA in vitro is specifically initiated at the third of three closely spaced AUG codons (position 623) in both the reticulocyte lysate and the wheat-germ extract (Herman, 1986, and unpublished observations). Initiation at the two in-phase upstream AUGs (positions 512 and 560) is not detected when in vitro or in vivo synthesized P mRNA is

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[1] Abbreviations: CBP, cap binding protein; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; M, matrix protein; N, nucleocapsid protein; ORF, open reading frame; P, phosphoprotein; SDS, sodium dodecyl sulfate; SSC, standard saline citrate buffer; VSV, vesicular stomatitis virus; UTP, uridine 5′-triphosphate.

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translated in either cell-free system. In contrast, translation of uncapped SP6 polymerase transcripts containing nucleotides 33–814 or 382–814 of the P mRNA is efficiently initiated at all three AUGs in this ORF (Herman, 1986).

Synthesis of the VSV 7K protein appears to be an exception to the scanning model for translation that has been proposed by Kozak [reviewed by Kozak (1983)]. The scanning model assumes that the ribosomes and associated factors must bind at or very near the capped 5′ end of the mRNA before they begin searching for a favorable AUG codon. This model predicts that hybrid arrest of the 5′ end of the mRNA should block access to all AUG codons regardless of their position in the sequence.

Translation of the 7K protein in vitro is unaffected by hybridization of the 5′ two-thirds of the full-length mRNA to an excess of a cloned (−)sense cDNA probe under conditions that abolish the synthesis of P. This rules out the possibility that the 7K protein is produced by premature termination of translation or by proteolytic cleavage of P. These results led me to propose a model in which ribosomes can bind and initiate translation internally on the P mRNA at a site located hundreds of nucleotides downstream from the capped 5′ end (Herman, 1986).

Recently published data suggest that the internal initiation model may also apply to the translation of several other viral mRNAs. This includes synthesis of a second protein from the adenovirus type 2 DNA polymerase mRNA (Hassin et al., 1986), synthesis of two of the three products encoded by the infectious pancreatic necrosis virus (a birnavirus) A segment (Nagy et al., 1987), and translation of the encephalomyocarditis virus mRNA (Shih et al., 1987).

Binding and initiation by ribosomes at an internal AUG codon suggests a number of testable predictions. The experiments presented here to test those predictions support this alternative model and imply that internal initiation is a distinct biochemical process.

Materials and Methods

Viral RNA. [^{3}H]Uridine-labeled VSV mRNA was extracted from suspension BHK cells at 4.5 h postinfection as previously described in detail (Herman, 1986). Viral mRNA was synthesized in vitro in a standard transcription reaction mixture containing [^{3}H]UTP (Herman, 1986). SP6 polymerase transcripts of plasmid pRH18 were prepared as outlined (Herman, 1986; Melton et al., 1984).

In Vitro Translation. Translations were performed in a reticulocyte lysate (Promega) exactly as described (Herman, 1986) or in a wheat-germ extract (Bethesda Research Laboratories) under conditions recommended by the supplier. Where noted, excess magnesium acetate or potassium acetate was added to the concentrations indicated in the figure legends. Translation products were immunoprecipitated with either polyclonal rabbit antiserum against the whole virus (provided by S. A. Moyer) or mouse monoclonal antibody against the carboxy terminus of P (provided by M. Williams and S. U. Emerson) as described (Herman, 1986). Proteins were electrophoresed in 17.5% polyacrylamide gels containing SDS (Laemmli, 1970) at 300 V until the tracking dye reached the bottom. Gels, fixed with acetic acid in methanol, were impregnated with Amplify (Amersham) and exposed at −70 °C. Bands were located by autoradiography, cut from the dried gel, hydrolyzed with H_2O_2, and counted in a liquid scintillation counter.

Hybrid-Arrested Translation. Hybrid-arrested translations were performed as previously described in detail (Herman, 1986) with P cDNA clones (Gallione et al., 1981) propagated as (−)sense single-stranded DNAs in M13 vectors.

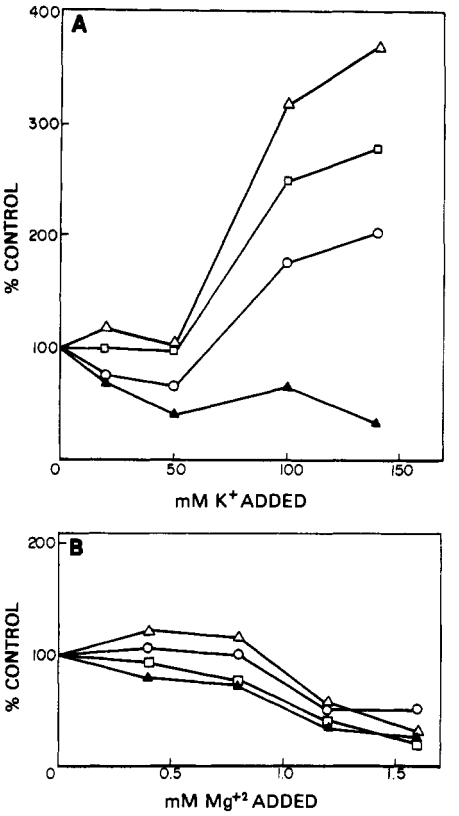

FIGURE 1: Ionic requirements for initiation. Replicate reticulocyte lysates programmed with the in vitro VSV mRNAs were incubated in the presence of increasing concentrations of either (A) potassium acetate or (B) magnesium acetate for 2 h at 30 °C. Small samples were precipitated with trichloroacetic acid to determine total incorporation of [^{35}S]methionine in each reaction. Portions of each lysate were immunoprecipitated with polyclonal rabbit antiserum directed against the virus and the proteins electrophoresed in a 17.5% polyacrylamide gel. Bands were cut out and counted in a liquid scintillation counter. The data are expressed as percent radioactivity in each band relative to the amount in the same band in the absence of exogenous ion. (○) Total acid-insoluble cpm (9681, 7105 cpm per μL of control lysate in the absence of added K^+ or Mg^{2+}, respectively); (□) N protein (8148, 7670 cpm in control gel band); (△) P protein (3911, 3946 cpm); (▲) 7K protein (1002, 1261 cpm).

Results

Ionic Requirements. To examine the requirements for mono- and divalent cations during the internal initiation of translation, small replicate reticulocyte lysates were programmed with in vitro poly(A)+ VSV mRNA in the presence of increasing amounts of either potassium acetate or magnesium acetate. The products were immunoprecipitated with polyclonal antiserum directed against the whole virus and resolved by electrophoresis in a polyacrylamide gel containing SDS. The bands were located by autoradiography and then cut out and counted.

Increasing the K^+ concentration over the endogenous level stimulated total incorporation of [^{35}S]methionine into acid-insoluble material as well as specific incorporation into viral nucleocapsid protein (N), P (Figure 1A), and matrix protein (M) (not shown). Stimulation of translation occurred at all excess K^+ concentrations tested here, and no inhibition of 5′ initiation was detected even in the presence of as much as 140 mM excess (Figure 1A).

In contrast, increasing the molarity of K^+ inhibited internal initiation (i.e., synthesis of the 7K protein) at all excess concentrations (Figure 1A). Internal initiation was decreased to

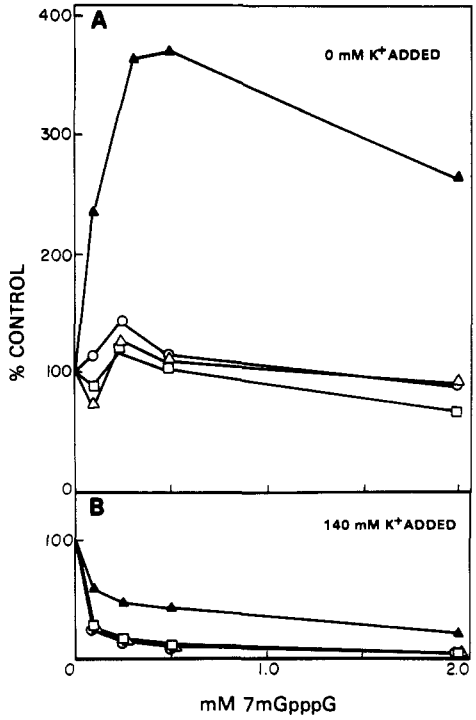

FIGURE 2: Effect of cap analogue on initiation in the reticulocyte lysate. Increasing amounts of the analogue 7mGpppG were added to identical reticulocyte lysates programmed with in vitro VSV mRNA. (A) Endogenous K^+ level; (B) 140 mM excess K^+ added. Data are expressed as in the legend to Figure 1. (○) Total acid-insoluble cpm (18883, 25861 cpm per μL of control lysate in (A) and (B), respectively); (□) N protein (15598, 30986 cpm in control gel band); (△) P protein (7575, 21279 cpm); (▲) 7K protein (1690, 352 cpm).

approximately 30% of the control level at the highest K^+ concentration tested (Figure 1A).

Increasing the Mg^{2+} concentration over the endogenous had only minimal effect on total, N, P (Figure 1B), or M (not shown) synthesis at low levels but ultimately inhibited at high concentrations. Nevertheless, Mg^{2+} inhibited internal initiation at all excess levels tested (Figure 1B). Ribosome binding and initiation at an internal AUG codon on the VSV P mRNA therefore has distinctive ionic requirements.

Effect of Cap Analogues. A prediction of the internal initiation model is that initiation at a site located hundreds of nucleotides downstream from the capped 5′ end of the mRNA may have a reduced requirement for the cap-binding protein (CBP). CBP is thought to play an important role in initiation at the 5′ end of eukaryotic mRNAs (Edery et al., 1983, 1984; Sonenberg, 1981; Sonenberg et al., 1981, 1983). Cap analogues have been shown to compete with capped messages under certain conditions for the limited supply of CBP and thereby inhibit the initiation of translation (Lodish & Rose, 1977; Weber et al., 1978).

At the endogenous K^+ level, increasing concentrations of the cap analogue 7mGpppG had essentially no effect on total incorporation or on the synthesis of N, P (Figure 2A), or M (not shown) in the reticulocyte lysate. This confirmed the previous report that these analogues did not inhibit 5′ initiation in the reticulocyte lysate at low concentrations of K^+ (Weber et al., 1978). Although the cap analogue had no effect on 5′ initiation under these conditions, there was a reproducible 3–4-fold stimulation of internal initiation. At the highest analogue concentration tested in this experiment (2 mM), internal initiation was still 2–3-fold higher than that at the control level (Figure 2A).

A low concentration of the cap analogue efficiently inhibited 5′ initiation in the reticulocyte lysate at 140 mM excess K^+.

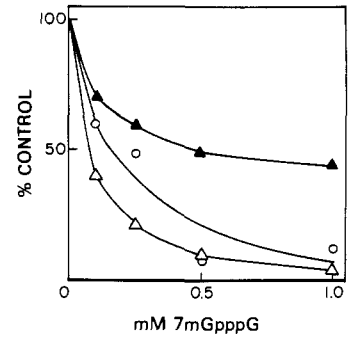

FIGURE 3: Effect of cap analogue on initiation in the wheat-germ lysate. Increasing amounts of analogue 7mGpppG were added to identical wheat-germ extracts programmed with in vitro VSV mRNA. Products were immunoprecipitated with a monoclonal antibody specific for the carboxy end of the P protein sequence. (○) Total acid-insoluble cpm (4288 cpm per μL of control lysate); (△) P protein (1235 cpm in control gel band); (▲) 7K protein (181 cpm).

The synthesis of N, P (Figure 2B), and M (not shown) was reduced to only about 5% of the control by the addition of 2 mM 7mGpppG.

Synthesis of the 7K protein showed a biphasic response to 7mGpppG in the presence of 140 mM excess K^+. Low concentrations of the analogue inhibited internal initiation by approximately 50%. This was followed by a much less sensitive phase, which remained at approximately 30–40% of the control level in the presence of 2 mM cap analogue. It should be noted that 140 mM excess K^+ was shown (Figure 1A) to be significantly inhibitory by itself for internal initiation.

Identical results were also obtained in a wheat-germ extract (Figure 3). Translation in wheat-germ extracts is sensitive to cap analogues at modest K^+ concentrations (Weber et al., 1978), and wheat-germ ribosomes have been reported to be more selective in their choice of start sites (Kozak & Shatkin, 1977). The products of the wheat-germ translation were analyzed in this experiment with a monoclonal antibody (supplied by M. Williams and S. U. Emerson) that is specific for the carboxy proximal sequence of the P protein (Herman, 1986).

In the presence of 1 mM 7mGpppG, synthesis of P was reduced to 4% of control in the wheat-germ extract, whereas synthesis of the 7K protein was reduced to approximately 50% of control (Figure 3). These results agree fully with those obtained in the reticulocyte lysate (Figure 2B). This experiment demonstrates that the more selective plant ribosomes can recognize the internal binding site, and it confirms that initiation at this site has a reduced requirement for CBP.

Translation of Expression Vector Transcripts. Translation of the pRH18 expression vector transcript (corresponding to nucleotides 382–814 of the P mRNA) initiates at two non-physiological start sites (positions 512 and 560) in addition to AUG_{623} in both the reticulocyte lysate and the wheat-germ extract (Herman, 1986). The resulting three overlapping translation products are nested at their carboxy termini and have apparent molecular weights of 13000, 9000, and 7000, respectively (Herman, 1986) (Figure 4b).

Hybrid arrest of the 5′-proximal portion of the uncapped transcript with a cloned (−)sense cDNA significantly altered the ratio of initiation at the three sites in the reticulocyte lysate. Under these conditions the 7K polypeptide, initiated at AUG_{623}, was the predominant product (Figure 4c). The 13K product was not detected, and only a trace of 9K product was visible (Figure 4c).

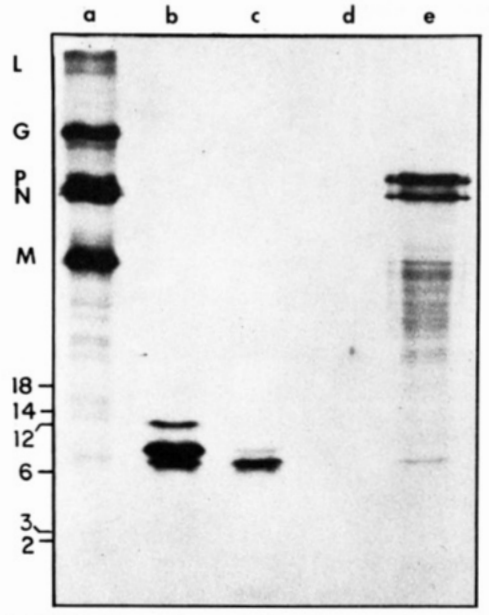

FIGURE 4: Hybrid-arrested translation of expression vector RNA. SP6 polymerase transcripts of the pRH18 expression vector were prepared as previously described (Melton et al., 1984). Hybrid-arrested translation in the reticulocyte lysate was with M13 vectors carrying the (−)sense strand of cloned VSV P cDNA fragments as described in detail (Herman, 1986). Products were immunoprecipitated with polyclonal antiserum directed against the virus prior to electrophoresis in a 17.5% polyacrylamide gel containing SDS. (a) ^{35}S-labeled VSV virion proteins, identified at left, without immunoprecipitation; (b) expression vector RNA incubated in the absence of added DNA prior to translation; (c) hybrid arrest with a cloned fragment complementary to nucleotides 127–533 of the P mRNA sequence; (d) hybrid arrest with a fragment complementary to nucleotides 534–814; (e) translation products of total in vitro VSV poly(A)+ mRNA. Approximately equal cpm of immunoprecipitated expression vector product was applied to lanes b and c. The locations of protein molecular weight standards, detected by Coomassie blue staining, are indicated at the left: (2) insulin A (2300); (3) insulin B (3400); (6) bovine trypsin inhibitor (6200); (12) cytochrome *c* (12 300); (14) lysozyme (14 300); (18) β-lactoglobulin (18 400).

Hybrid arrest of the 3′ end of the expression vector transcript with a cloned (−)sense cDNA abolished all translation and thereby demonstrated that the products were VSV-specific (Figure 4d). These results suggested that AUG_{512} and AUG_{560} were probably accessed by scanning from the 5′ end of the expression vector transcript and that AUG_{623} could be accessed by internal binding.

Effect of Cap Analogue on Expression Vector Transcripts. The ability of ribosomes to initiate at three sites on the expression vector transcript (Figure 4) afforded an opportunity to examine the effects of the cap analogue on simultaneous initiation at all three internal AUGs. These start codons are located 130, 178, and 241 nucleotides, respectively, from the 5′ end of the pRH18 expression vector transcript.

Increasing concentrations of 7mGpppG significantly inhibited translation of the expression vector transcript in the wheat-germ extract (Figure 5). Initiation at AUG_{512} and AUG_{560} was reduced approximately 10-fold by the highest analogue concentration, whereas initiation at AUG_{623} was reduced only about 2.5-fold. These reductions were similar in magnitude to those observed for 5′-proximal and internal initiation, respectively, on the capped full-length P mRNA both in the reticulocyte lysate (Figure 2B) and in the wheat-germ extract (Figure 3). Thus, even though efficient initiation could be induced at the two upstream AUGs, the ribosomes and associated factors were still able to distinguish those codons from the correct internal start site at position 623 when assayed in the presence of the cap analogue.

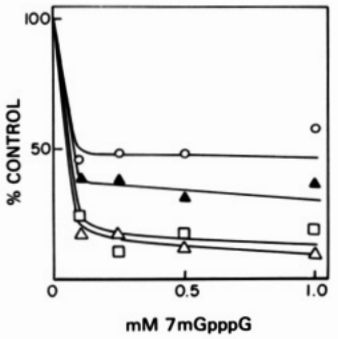

FIGURE 5: Effect of cap analogue on initiation on expression vector RNA. The SP6 polymerase transcript of plasmid pRH18 was translated in the wheat-germ extract in the presence of increasing amounts of 7mGpppG. Data are expressed as described in the legend to Figure 1. The average values from two gels are plotted. (○) Total acid-insoluble cpm (2441 cpm per μL of control lysate); (△) 13K product (25259 cpm in control gel band); (□) 9K product (31565 cpm); (▲) 7K product (5175 cpm).

DISCUSSION

The results presented here support the previously proposed model (Herman, 1986) for the internal initiation of translation. Experiments to determine the ionic optima and the effects of the cap analogue show that 5′-proximal and internal initiations have different requirements. That both animal and plant translation systems recognize AUG_{623} and respond similarly to cap analogue (albeit at different K^+ concentrations) implies that internal initiation is not simply an artifact of false starts and false products. Other preliminary experiments also suggest that the P mRNA may bind two ribosomes in the presence of a high concentration of anisomycin (unpublished results).

Even though initiation occurs at all three internal AUGs on the expression vector transcript, the translation apparatus can nevertheless distinguish the authentic internal start site from the others in the presence of cap analogue. These results attest to the uniqueness of AUG_{623} and support the conclusion that 5′-proximal and internal initiations are separate and distinct biochemical processes.

Stimulation of internal initiation in the reticulocyte lysate by the addition of the cap analogue at the endogenous K^+ concentration may reflect the normal regulation of initiation at the two sites. At a low concentration of mRNA (i.e., cap structures) 5′-proximal initiation appears to be the favored reaction. However, at high concentration, saturation of some limiting factor may shift the normal equilibrium and thereby increase internal initiation.

The partial inhibition of internal initiation induced by the cap analogue in the wheat-germ extract or at high K^+ in the reticulocyte lysate suggests that CBP (or perhaps another cap-sensitive component) has a function, beyond recognition of the 5′ end, that is important for both 5′-proximal and internal initiations. Indeed, the ability of cap analogue to affect initiation at all three AUGs on the pRH18 expression vector transcript supports this suggestion.

Previously reported data suggest that approximately 5–10 molecules of P are synthesized per molecule of 7K protein (Herman, 1986). If the rates of protein synthesis (initiation plus elongation) of the two products were exactly equal, then only 1 molecule of 29K P protein should be synthesized per 4 molecules of 7K protein. It is unlikely that the elongation rate along the 3′ portion of the P mRNA would be affected by the location of the start codon. Consequently, the rate-limiting difference may be the efficiency of ribosome binding

or initiation at the capped 5′ end relative to that at the internal site.

Additional experiments will be required to elucidate the potential role of CBP and other initiation factors in the internal initiation of translation. Preliminary in vitro experiments suggest that internal initiation may be somewhat less sensitive to the polio virus induced cleavage of the CBP complex when assayed in extracts of polio-infected HeLa cells (R. Herman and R. Lundquist, unpublished observations). The function of the 7K protein, which may be a minor component of the VSV virion (unpublished observations), also remains to be determined.

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Registry No. 7mGpppG, 110971-03-4; K, 7440-09-7; Mg, 7439-95-4.

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Demonstration That Lysine-501 of the α Polypeptide of Native Sodium and Potassium Ion Activated Adenosinetriphosphatase Is Located on Its Cytoplasmic Surface†

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ABSTRACT: Evidence that the peptide HLLVMKGAPER, which can be released from intact sodium and potassium ion activated adenosinetriphosphatase by tryptic digestion, is located on the cytoplasmic surface of the native enzyme has been obtained. An immunoadsorbent directed against the carboxy-terminal sequence of this tryptic peptide has been constructed. The peptide KGAPER was synthesized by solid-phase techniques. Antibodies against the sequence -GAPER were purified by immunoadsorption, using the synthetic peptide attached to agarose beads. These antibodies, in turn, were coupled to agarose beads to produce an immunoadsorbent. Sealed, right-side-out vesicles, prepared from canine kidneys, were labeled with pyridoxal phosphate and sodium [^{3}H]borohydride in the absence or presence of saponin, respectively. A tryptic digest of these labeled vesicles was passed over the immunoadsorbent. Large increases in the incorporation of radioactivity into the peptides bound by the immunoadsorbent were observed in the digests obtained from the vesicles exposed to saponin. From the results of several control experiments examining the labeling reaction as applied to these vesicles, it could be concluded that this increase in incorporation resulted only from the access that the reagents gained to the inside of the vesicles in the presence of saponin and that the increase in the extent of modification was due to the cytoplasmic disposition of this segment in the native enzyme.

The general problem of identifying membrane-spanning sequences in membrane-spanning proteins is supposed to have both a computational solution and an experimental solution. Although several other methods have been proposed, which differ in detail (Argos et al., 1982; Guy, 1984; Kuhn & Leigh, 1985; Eisenberg et al., 1984; Engelman et al., 1986), our own algorithm (Kyte & Doolittle, 1982) is illustrative of the computational solution. Each amino acid in a given segment 19 residues in length is assigned a numerical value reflecting its

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