JOURNAL OF VIROLOGY, Apr. 1989, p. 1651–1660
0022-538X/89/041651-10$02.00/0

Vol. 63, No. 4

Initiation of Protein Synthesis by Internal Entry of Ribosomes into the 5′ Nontranslated Region of Encephalomyocarditis Virus RNA In Vivo

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Received 7 November 1988/Accepted 15 December 1988

Expression vectors that yield mono-, di-, and tricistronic mRNAs upon transfection of COS-1 cells were used to assess the influence of the 5′ nontranslated regions (5′NTRs) on translation of reporter genes. A segment of the 5′NTR of encephalomyocarditis virus (EMCV) allowed translation of an adjacent downstream reporter gene (CAT) regardless of its position in the mRNAs. A deletion in the EMCV 5′NTR abolishes this effect. Poliovirus infection completely inhibits translation of the first cistron of a dicistronic mRNA that is preceded by the capped globin 5′NTR, whereas the second cistron preceded by the EMCV 5′NTR is still translated. We conclude that the EMCV 5′NTR contains an internal ribosomal entry site that allows cap-independent initiation of translation. mRNA containing the adenovirus tripartite leader is also resistant to inhibition of translation by poliovirus.

The scanning hypothesis of eucaryotic protein synthesis (25, 26) postulates that the initial contact between protein components (initiation factors and 40S ribosomal subunit) and mRNA occurs at the 5′ end of the polynucleotide chain. Here we report that translation of RNAs controlled by the 5′ nontranslated region (5′NTR) of encephalomyocarditis virus (EMCV) does not follow this rule in vivo.

The most compelling argument favoring the scanning hypothesis rests on the realization by Jacobson and Baltimore in 1968 that eucaryotic mRNAs are likely to encode only one polypeptide (19). Indeed, no functional polycistronic cellular mRNA with nonoverlapping cistrons has been identified to date. Certain mRNAs of mammalian viruses, however, are translated into two distinct proteins, but in these instances translation usually occurs from overlapping reading frames (61). Most eucaryotic mRNAs are translated from the first AUG, and eucaryotic ribosomes may not normally bind to mRNAs at internal AUG codons, that is, hundreds or thousands of nucleotides (nt) downstream from the 5′ end, as is the case in translation of procaryotic mRNA. The discovery of the 5′-terminal cap structure (m^7GpppN) of eucaryotic mRNA and the description of proteins with the ability to bind both to the cap structure and to ribosomal subunits provided a plausible mechanism by which the 40S ribosomal subunits could be attracted to the 5′ end of mRNA (60, 61). However, initiator AUG codons are seldom located immediately adjacent to the cap structure. It therefore follows that an initiation complex formed at the 5′ end must migrate along the 5′NTR to the initiating AUG. Kozak and her colleagues called such migration of the 40S subunits along the nucleotide chain "scanning." Many observations concerning protein synthesis in vitro and in vivo can be satisfactorily explained by the scanning hypothesis (25, 26). Comparison of eucaryotic mRNA sequences and the results of site-directed mutagenesis experiments (29) have demonstrated the existence of a preferred nucleotide context surrounding the initiation codon (A/GccAUGG). However, the mechanism of selection of the initiation codon is unclear, particularly if there is an unused AUG in a favorable context preceding the real initiation codon (28).

Picornavirus mRNAs are genomic RNAs of about 7,500 nt from which the 5′-terminal oligopeptide (VPg) has been removed (17, 38, 39, 50). They are unique in that their 5′ ends are terminated by monophosphate residues (pUpU. . . .). The attachment of a ribosomal subunit to such a terminus, which is distinct from the cap structure, may be very inefficient or may not occur at all. Translation of picornavirus mRNAs is efficient in infected cells, however, and results in the rapid and highly productive life cycle of these viruses. Moreover, the picornaviral RNAs are efficiently translated in cell-free protein synthesis systems (14, 16). Other distinct features of picornavirus mRNAs are the extended length of their 5′NTRs (650 to 1,300 nt) and the number of unused AUG codons (6 to 13) preceding the initiator codon for the polyprotein (43; A. Palmenberg, personal communication). The function(s) of these long 5′NTRs is unknown, but viral replication is impaired even by minor structural changes in this region, such as point mutations, linker insertions, or small deletions (12, 33, 40, 47, 52, 55, 62). The 5′NTRs of *Cardiovirus* and *Aphthovirus* (two genera of *Picornaviridae*) are further distinct in that they contain a segment of poly(C). For example, EMCV, a cardiovirus, contains a 5′NTR that is 833 nt long with the poly(C) tract located between nt 149 and 263. Although 10 AUGs are scattered throughout the EMCV 5′NTR in all three reading frames, initiation of the viral polyprotein commences at the 11th AUG (44).

In light of the unusual properties of picornavirus mRNAs, we have asked whether the 40S ribosomal subunit must bind to the 5′-terminal pU of picornavirus mRNA and scan along the 5′NTR for initiation of protein synthesis. Previous experiments on translation of dicistronic mRNAs in vitro strongly suggested that the EMCV 5′NTR allows "internal" binding of ribosomes and translation of a downstream reporter gene (20). Translation of picornavirus mRNA in vitro, however, may discriminate against conventional 5′ scanning because components involved in this mechanism may be in short supply in cell extracts (9). We have therefore con-

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structed expression vectors that produce multicistronic mRNAs upon transfection in COS-1 cells, and we have measured the expression of an indicator gene (CAT) under the control of EMCV 5'NTR. While this manuscript was in preparation, Pelletier and Sonenberg informed us that they have performed similar experiments, but these investigators used the 5'NTR of poliovirus (49). Our data with the 5'NTR of EMCV concur with their results in that internal ribosomal binding to the viral 5'NTR can occur in poliovirus-infected cells in which cap-dependent initiation of protein synthesis is inhibited. In addition, we have extended these studies by analyzing the translation of multicistronic mRNAs in cells not impaired by poliovirus infection. We conclude that the picornavirus 5'NTR facilitates a special mechanism of initiation of translation. Other viral mRNAs, such as that of adenovirus, may use such a mechanism also.

MATERIALS AND METHODS

Construction of plasmids directing transcription of mono-, di-, and tricistronic mRNAs in vivo. Enzymes used in cloning and modifying DNAs were purchased from New England BioLabs, Inc., and Bethesda Research Laboratories, Inc. DNA manipulations were done by standard procedures (36).

Plasmid pBS-ECAT was constructed by ligation of pBS(+) (Vector Cloning Systems), digested with *Eco*RI and *Pst*I, to a 590-base-pair fragment of pE5-LVP0 (from the *Eco*RI site to the Klenow-filled *Nco*I site; 32) and to a 2,175-base-pair fragment of pSV2-CAT (from the Klenow-filled *Hin*dIII site to the *Pst*I site; 15). T7 transcripts of this plasmid contain the 5'NTR of EMCV from nt 260 to 848, which includes the putative internal ribosomal entry site (IRES) identified by in vitro study (20) and the authentic initiation codon of the EMCV polyprotein. The EMCV initiation codon was placed in frame with the authentic initiation codon of the chloramphenicol acetyltransferase gene (CAT) gene. Translation from the initiation codon of EMCV RNA would produce a polypeptide (CAT') 17 amino acids longer than the bacterial CAT protein translated from the initiation codon of the CAT gene.

The eucaryotic expression vector pMT2 was derived from p91023B (65) by inserting the eucaryotic transcription unit (*Xho*I-*Bgl*II) of p91023B into pUC18 which had been previously modified to include the simian virus 40 (SV40) origin of replication (nt 5,172 to 273) and the adenovirus type 2 VAI gene (nt 10,229 to 10,813). Plasmid pMT2-ADA was derived from pMT2 by inserting the cDNA of human adenosine deaminase (ADA) into the *Eco*RI site of pMT2. Plasmid pMT2-ECAT1 was constructed from pBS-ECAT and pMT2-ADAΔEco3', that is, a derivative of pMT2-ADA from which an *Eco*RI site located after the ADA gene had been removed (Fig. 1; see reference 24). The 5'NTR of EMCV (nt 260 to 848) and the CAT gene of pBS-ECAT were placed into the *Eco*RI site of pMT2-ADAΔEco3' by insertion of a linker (3'-ACGTCCGCTCGGACTTAA-5') into the *Pst*I site of a 2,656-base-pair fragment generated from pBS-ECAT with *Eco*RI and *Pst*I. The resulting plasmid (pMT2-ECAT1; Fig. 1A) directs transcription of MT2-ECAT1 mRNA (MT2-ECAT1; Fig. 1B), which is monocistronic because a polyadenylation signal (originating from pSV2-CAT) is located after the CAT gene. Plasmid pMT2-ECATΔ1 was constructed similarly to pMT2-ECAT1, except that a *Pst*I–Klenow-filled *Hin*dIII fragment from pBS-ECAT was used instead of the *Pst*I-*Eco*RI fragment from pBS-ECAT. This plasmid directs transcription of a monocistronic MT2-ECATΔ1 mRNA in which part or all of the putative IRES is deleted (nt 260 to 484 in the 5'NTR of EMCV RNA).

Plasmids pMT2-ECAT2 and pMT2-ECATΔ2 were constructed similarly to pMT2-ECAT1 and pMT2-ECATΔ1, respectively, except that pMT2-ADAΔEco5' (a derivative of pMT2-ADA from which an *Eco*RI site located before the ADA gene had been removed) was used instead of pMT2-ADAΔEco3'. Plasmid pMT2-ECAT2 directs transcription of a dicistronic mRNA containing the ADA cistron and the ECAT cistron sequentially from 5' to 3' end (Fig. 1). Plasmid pMT2-ECATΔ2 also directs transcription of a dicistronic mRNA but has an additional deletion (nt 260 to 484) in the 5'NTR of EMCV.

Plasmid pMT2-ECAT3 was constructed from pMT2-ADAΔEco3' and pBS-ECAT. Plasmid pMT2-ADAΔEco3' (cut with *Hpa*I) was ligated with a fragment of pBS-ECAT (from the *Eco*RI site to the Klenow-filled *Hin*dIII site) and with an *Eco*RI linker (5'-AATTCCTCGAGAGC-3'). Plasmid pMT2-ECAT3 (Fig. 1A) directs transcription of a tricistronic mRNA which contains the ADA, the DHFR (dihydrofolate reductase), and the ECAT cistrons in the first, second, and third positions in the mRNA, respectively (Fig. 1B). Plasmid pMT2-ECATΔ3 was constructed similary to pMT2-ECAT3, but a Klenow-filled *Hin*dIII fragment of pBS-ECAT was used to provide the ECAT cistron (Fig. 1A). The transcript of this plasmid is a tricistronic mRNA with the same deletion as in MT2-ECATΔ1 (Fig. 1B).

Plasmid pβ-ECAT2 was constructed by a three-fragment ligation of a Klenow-filled *Eco*RI fragment of pβe$^-$ (41), an *Nde*I–Klenow-filled *Bam*HI fragment of pMT2-ECAT2, and an *Nde*I-Klenow-filled *Bgl*II fragment of pMT2-ECAT2. This plasmid contains the promoter and the 5'NTR of the human β-globin gene (see Fig. 5). Note that all the other plasmids used in this experiment contain the adenoviurs tripartite leader at the 5' termini of the transcripts (Fig. 1). The transcript of pβ-ECAT2 is a dicistronic mRNA in which the first β-globin/ADA hybrid cistron is expressed under the translational control of the β-globin leader sequence, whereas the translation of the second ECAT cistron is controlled by the leader sequence of EMCV RNA.

Analysis of expression of mono-, di-, and tricistronic vectors. COS-1 cells were transfected using DEAE-dextran and an additional chloroquine treatment (24, 35, 59). Synthesis of the ECAT, ADA, and DHFR gene products was monitored 48 h posttransfection (p.t.) by labeling 2×10^6 COS-1 cells for 1 h with 100 μCi of L-[^{35}S]methionine (specific activity, >10,000 Ci/mmol; New England Nuclear Corp.). Cell extracts were prepared and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) either before or after immunoprecipitation with a sheep anti-mouse ADA antibody, a rabbit anti-mouse DHRF antibody, or a monoclonal anti-CAT primary antibody and a rabbit anti-mouse immunoglobulin G (IgG) secondary antibody. Protein A-Sepharose CL-4B (Pharmacia) was used as an immunoadsorbent. Gels were fixed in 30% methanol–10% acetic acid–10% trichloroacetic acid, prepared for fluorography by treatment with En^{3}Hance (New England Nuclear Corp.), and dried. Dried gels were autoradiographed with Kodak XAR-5 film.

Assay of CAT activity in COS-1 cells was done by the method of Gorman et al. (15). Extracts of 2×10^6 cells were prepared 48 h p.t. by three cycles of freeze-thawing in 200 μl of 0.25 M Tris hydrochloride (pH 7.8) and centrifugation for 15 min in an Eppendorf centrifuge at 4°C. The supernatants were assayed in a mixture containing 150 μl of 0.25 M Tris hydrochloride (pH 7.8), either 0.04 or 2 optical density units (at 595 nm) of cell extract (determined by using a BioRad assay), 1 μCi of [^{14}C]chloramphenicol (New England Nu-

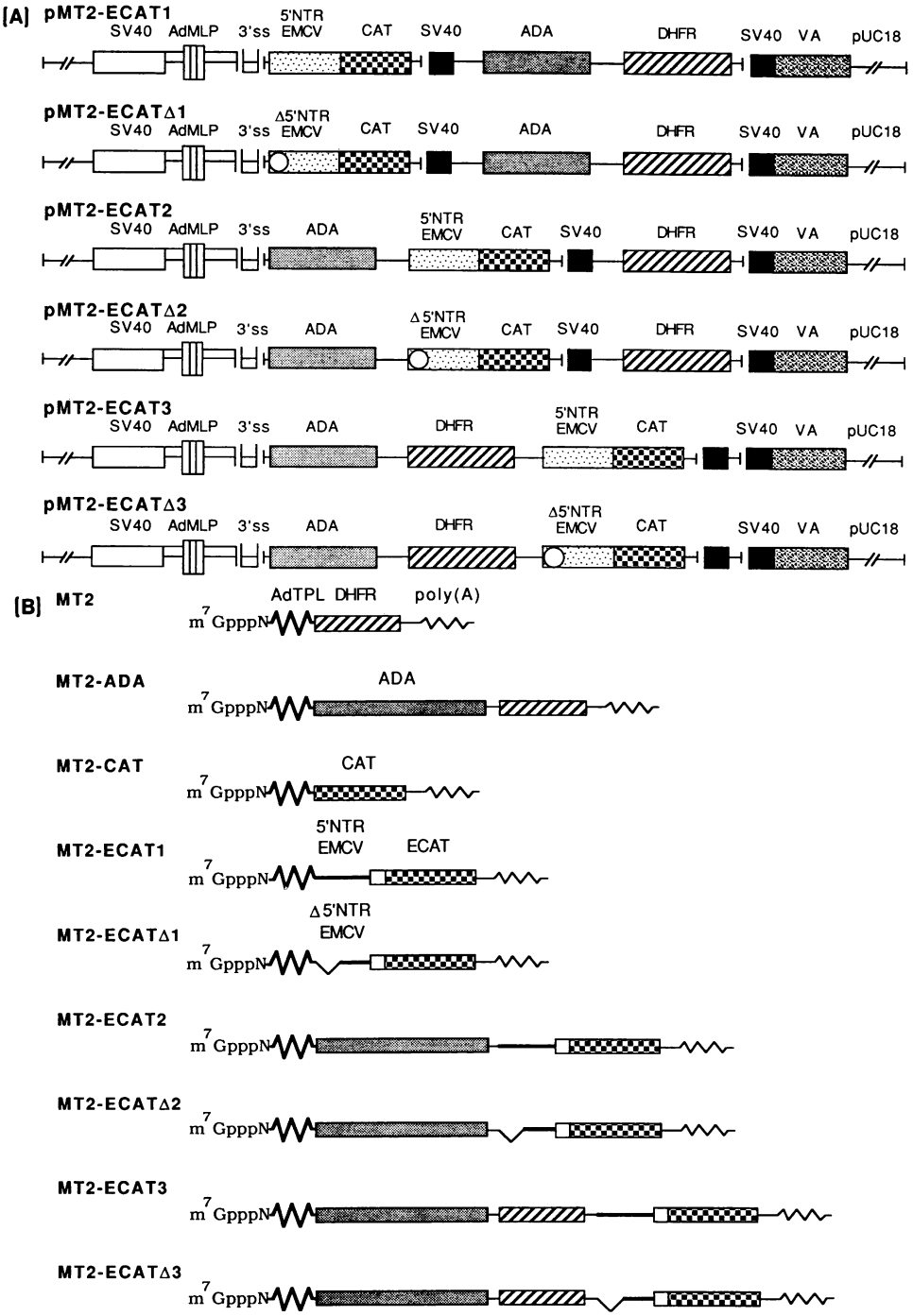

FIG. 1. Structure of polycistronic expression vectors and their mRNA transcripts. (A) The functional elements of the expression vectors have been described previously (24). All vectors utilize the adenovirus major late promoter (AdMLP) for transcription initiation and contain the majority of the adenovirus tripartite leader present in adenovirus late mRNAs. A small intron is present which consists of a 5′ splice site, originating from the adenovirus first late leader, and a 3′ splice site (3′ss). In addition, the vectors contain the SV40 origin of replication and tanscriptional enhancer, which are shown as open boxes; one or two SV40 early polyadenylation signals, which are shown as solid boxes; and the adenovirus VA genes (VA). The human ADA, murine DHFR, and bacterial CAT coding regions are located as indicated. The 5′NTR of EMCV is depicted as a dotted box, and its deletion derivative is shown as a dotted box containing an open circle. Figures are not drawn to scale. (B) mRNAs transcribed from the vectors shown in panel A. The cap structure (m^7GpppN, where N is any nucleotide) is followed immediately by the adenovirus tripartite leader (thick zig-zag line) and various cistrons. A line between the coding sequences (shown as boxes) represents the 5′NTR and the 3′NTR of the coding sequences. The thick straight line represents the 5′NTR of EMCV (nt 260 to 833), or the Δ5′NTR of EMCV (nt 485 to 833) where the additional deletion (nt 260 to 484) is indicated (⌒). All mRNAs are polyadenylated (thin zig-zag line). The hatched, shaded, and checked boxes represent DHFR, ADA, and CAT cistrons, respectively. The open box which precedes the CAT cistron represents an additional 17-amino-acid-coding sequence which starts at the initiation codon of the EMCV polyprotein, in frame with the CAT gene. The 5′NTRs of DHFR and ADA are 99 and 69 nt long, respectively, and contain no AUG codon. The truncated 5′NTR of EMCV used here is 574 nt long and contains nine AUGs. The Δ5′NTR of EMCV is 350 nt long and contains five AUGs. The 3′NTR of DHFR is 187 nt long and contains five AUGs. The 3′NTR of ADA is 350 nt long and contains six AUGs.

clear Corp.), and 20 μl of 4 mM acetyl coenzyme A. The reaction mixture was incubated for 20 min at 37°C. The reaction was stopped by addition of 1 ml of cold ethyl acetate, which was also used to extract the chloramphenicol. Different forms of acetylated chloramphenicol were separated by silica-gel thin-layer chromatography.

Total RNA was prepared by guanidine thiocyanate extraction (4) and was examined by Northern (RNA) blot hybridization (63) following electrophoresis in formaldehyde-formamide denaturing 1% agarose gels and transfer to nitrocellulose (7). Hybridization was carried out using gel-isolated restriction fragments which had been labeled with [α−^{32}P]dATP by nick translation (24).

mRNA levels of transfected plasmids were determined by primer extension of RNAs purified from transfected cells. An oligodeoxyribonucleotide synthesized complementary to nt 48 to 64 of the transcripts was used as a primer. Northern blot analysis was also done to assess the mRNA level of MT2-CAT (data not shown). Autoradiographs were analyzed by densitometry. The relative translational efficiency of ECAT cistrons in different transcripts as measured by CAT activity was normalized with respect to the mRNA level derived from each construct as measured by primer extension.

Expression of dicistronic vectors in poliovirus-infected cells. COS-1 cells transfected with pβ-ECAT2 or pMT2-ECAT2 by the DEAE-dextran–chloroquine method were infected 48 h p.t. with poliovirus type 1 (Mahoney) at a multiplicity of infection of 100 PFU/cell. Expression of gene products from the two vectors was monitored by pulse-labeling 2 × 10^6 cells with [^{35}S]methionine (100 μCi) for 30 min at 0, 3, or 4 h postinfection (p.i.). The labeled cell extracts were analyzed by SDS-PAGE either before or after immunoprecipitation with anti-ADA or anti-CAT antibodies as described.

RESULTS

Translational efficiencies of different cistrons in mono-, di-, and tricistronic mRNAs. We have tested whether internal entry of ribosomes within the 5′NTR of EMCV occurs in vivo by measuring the expression of a chloramphenicol acetyltransferase gene (CAT gene; 56) connected to the EMCV sequence from nt 260 to 848. We will refer to the coding region of the EMCV/CAT construct as ECAT. Previously we have shown that the EMCV 5′NTR deleted up to nt 259 directs translation in vitro as efficiently as does the full-length EMCV 5′NTR (20), and for brevity we will refer to this truncated viral leader as EMCV 5′NTR. Ligation of the CAT gene to the EMCV 5′NTR sequence resulted in synthesis of a hybrid protein (CAT′) composed of the N-terminal 5 amino acids of the EMCV polyprotein, 12 amino acids encoded by the sequence immediately preceding the CAT gene, and the CAT protein. The ECAT element was placed in different positions in the vector pMT2-ADA (Fig. 1A), and the vectors were introduced into COS-1 cells by transfection, where they produced mono-, di-, and tricistronic mRNAs (Fig. 1B). For increased gene expression in a transient transfection experiment (23, 24), the vectors contained the adenoviurs VA genes, the adenovirus major late promoter, the majority of the adenoviurs tripartite leader, a small intron containing a 5′ splice site and an introduced 3′ splice site, the SV40 polyadenylation signal, and the SV40 origin of replication to allow replication in COS-1 cells (13).

Genes preceding ECAT in the expression vectors were either the human adenosine deaminase gene (ADA gene; 42) in pMT2-ECAT2 or the ADA gene followed by the dihydrofolate reductase gene (DHFR gene; 22; refer to Fig. 1A). Two monocistronic vectors (pMT2 and pMT2-CAT) and a dicistronic vector (pMT2-ADA) were used to measure expression of genes in the absence of ECAT (see Fig. 1).

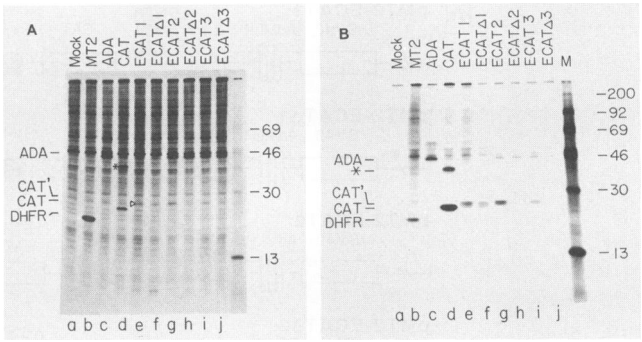

FIG. 2. Analysis of [^{35}S]methionine-labeled proteins synthesized in transfected COS-1 cells. (A) SDS-PAGE of total proteins that were labeled with [^{35}S]methionine for 1 h at 48 h p.t. The lanes are labeled according to the mRNA species produced in the transfected cells, although the notation MT2 is used only for the second lane. Lane d contains the CAT band and an additional vector-induced band of unknown identity (indicated by an asterisk). The CAT′ band migrated slightly more slowly than the CAT band because it is 17 amino acids longer. The last lane contains ^{14}C-labeled marker proteins. (B) Analysis of immunoprecipitated CAT proteins. The labeled polypeptides were immunoprecipitated with a monoclonal anti-CAT antibody as described in Materials and Methods and analyzed by SDS-PAGE. The lanes are labeled as for panel A. Several vector-encoded polypeptides were precipitated by sandwich immunoprecipitation. Nonspecific precipitation is probably due to the secondary rabbit anti-mouse IgG antibody as discussed in the text.

Transfection of COS-1 cells with these plasmids yielded polypeptides that could be detected 48 h p.t. by pulse-labeling cells with [^{35}S]methionine and analyzing total cell lysates by SDS-PAGE (Fig. 2A) or by immunoprecipitation (Fig. 2B). Note that the expression of DHFR from plasmid pMT2 (Fig. 2A, lane b) was strong, whereas it was absent in cells transfected with pMT2-ADA (Fig. 2A, lane c), a vector in which the DHFR cistron is in the second position. ADA, on the other hand, was strongly expressed from pMT2-ADA (Fig. 2, lanes c), as expected from its position in the dicistronic vector. The CAT gene product from vector pMT2-CAT, with a leader identical to that in pMT2-ADA, was also strongly expressed (Fig. 2, lanes d).

Expression of the ECAT cistrons from different vectors is shown (Fig. 2, lanes e, g, and i). The CAT′ product (open triangle in Fig. 2A, lane e), whose identity was confirmed by immunoprecipitation (Fig. 2B), migrated slightly more slowly than did the authentic CAT gene product (lanes d) and comigrated with a band seen also in mock-infected cells (Fig. 2A, lane a). The apparent increased molecular weight of CAT′ is most likely due to initiation of translation at the authentic initiation codon of the EMCV polyprotein, yielding a polypeptide 17 amino acids longer than the bacterial CAT enzyme. We cannot explain the nonspecific immunoprecipitations in Fig. 2B (lanes b and c), but in later experiments we could greatly reduce these by preadsorption of cell extracts with the rabbit anti-mouse IgG antibody (see Fig. 6B). Immunoprecipitation using an anti-DHFR antibody confirmed the identity of DHFR in pMT2-transfected cells. However, DHFR was not detected by immunoprecipitation in pMT2-ECAT3-transfected cells (data not shown), an observation corroborating previous studies by Kaufman et

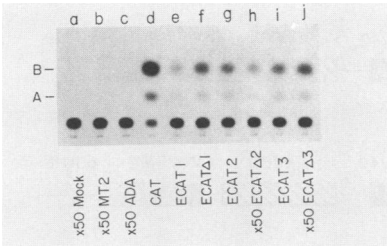

FIG. 3. Assay of CAT activity in vector-transfected COS-1 cells. The CAT assay was done as described in Materials and Methods at 48 h p.t., using two different amounts of cell extracts. Chloramphenicol and its acetylated forms, 1-acetate chloramphenicol (A) and 3-acetate chloramphenicol (B), were detected by autoradiography after thin-layer chromatography.

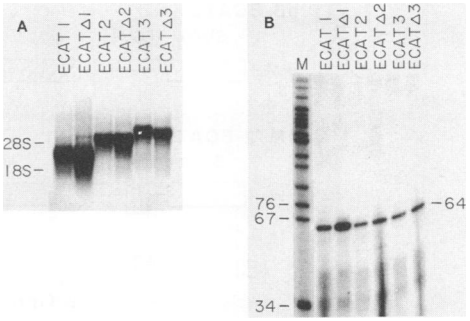

FIG. 4. Stability and level of mono- and polycistronic mRNAs. (A) Northern blot analysis of mRNAs. Total RNA of transfected COS-1 cells was isolated and electrophoresed on a formaldehyde-formamide denaturing 1% agarose gel, transferred to a nitrocellulose filter, and hybridized with a ^{32}P-labeled fragment of the CAT-coding region. The locations of 18S and 28S rRNAs are indicated. Lanes are labeled as for Fig. 2A. (B) Primer extension of mRNAs. Total RNA was isoalted from transfected COS-1 cells, and primer extensions were performed as described in Materials and Methods. The lanes are labeled as for Fig. 2A. Lane M contains size marker DNAs prepared by ^{32}P-phosphorylation of 5′ ends of *Msp*I-digested pBR322 fragments.

al. (24). We conclude that the ECAT cistron was expressed regardless of whether it was located in position 1, 2, or 3 of mono-, di-, or tricistronic mRNAs. The difference in expression of ECAT and DHFR cistrons is likely to be due to the properties of the 5′NTRs preceding their respective coding regions.

We have recently observed that deletion up to nt 484 of the EMCV 5′NTR (referred to as Δ5′NTR of EMCV) abolishes the ability of the leader to accommodate internal ribosome binding in vitro (20). Accordingly, we constructed derivatives of the pMT2-ECAT plasmids with an additional deletion in the EMCV 5′NTR from nt 260 to 484 (referred to as ECATΔ; see Fig. 1). Although there was some synthesis of CAT′ in the monocistronic mRNA MT2-ECATΔ1 (Fig. 2, lane f), the deletion reduced expression of the ECAT cistron in di- and tricistronic mRNAs below levels detectable by immunoprecipitation (Fig. 2B, lanes h and j, respectively). This apparent reduction was not due to decreased efficiency of transfection or transcription, since the ADA gene product was synthesized in equal amounts from pMT2-ECAT2, -ECATΔ2, -ECAT3, and -ECATΔ3 (Fig. 2A, lanes g through j). The decreased expression of CAT′ from these deletion constructs did not result from mRNA instability or from mRNA modification (see below). We attribute the synthesis of CAT′ in the monocistronic mRNA to the presence of the strong adenovirus tripartite leader in the 5′NTR preceding the deleted ECAT.

CAT activity in transfected cells. Expression of CAT enzyme from various vectors was verified by measuring the conversion of chloramphenicol to its 1-acetate and 3-acetate derivatives (15). As expected, there was no CAT activity in mock-, pMT2-, and pMT2-ADA-transfected cells (Fig. 3, lanes a through c). CAT activity was apparent in all cells transfected with plasmids containing the ECAT cistron (Fig. 3, lanes e through j), regardless of the position of this cistron within the di- and tricistronic mRNAs. However, CAT activity in extracts of cells transfected with pMT2-ECATΔ2 or pMT2-ECATΔ3 was detected only when 50 times more extract was used (Fig. 3, lanes h and j). Somewhat to our surprise, expression of the ECAT cistron from monocistronic mRNA was slightly greater when the cistron was preceded by the Δ5′NTR of EMCV (Fig. 3, lane f) than when it was preceded by the 5′NTR of EMCV (lane e). As will be shown below, this may be due in this experiment to the increased mRNA level from plasmid pMT2-ECATΔ1.

Stability and level of vector-specific mRNAs and CAT activities. It could be argued that the polycistronic transcription units yielded alternate mRNAs or that the transcripts were degraded to yield mRNAs that were effectively monocistronic. Such events could lead to the observed expression of the ECAT cistron in position 2 or 3 of polycistronic mRNAs. We therefore analyzed the size of the mRNAs by Northern blot analysis (63), using ^{32}P-labeled DNA containing a segment of the CAT gene as a probe. The size of the mRNAs detected in different extracts increased in relation to the size of the transcriptional units of different vectors (Fig. 4A). No RNA species comigrating with the monocistronic mRNAs (Fig. 4A, lanes ECAT1 and ECATΔ1) were apparent in cells transfected with pMT2-ECAT2, -ECATΔ2, -ECAT3, or -ECATΔ3, which strongly suggests that the CAT′ proteins produced in cells transfected with pMT2-ECAT2 and pMT2-ECAT3 were not translated from alternate or degraded transcripts.

We also performed primer extension analyses, using an oligodeoxyribonucleotide primer complementary to the sequence nt 48 to 64 of the vector-derived mRNAs, to assess the level of mRNA derived from each vector in transfected cells. Roughly equal amounts of mRNAs were produced from all vectors containing ECAT (Fig. 4B). An exception was pMT2-ECATΔ1, which yielded more primer extension product. This was reflected in the Northern blot analysis, in which pMT2-ECATΔ1-transfected cells contained more vector-specific mRNA than the others (Fig. 4A). We estimated the levels of mRNAs and CAT activities from densitometer tracings of the film shown in Fig. 3 and 4. When the CAT activities found in cells transfected with pMT2-ECAT1 and -ECATΔ1 were divided by the amounts of respective mRNAs observed either by Northern blot analysis or by primer extension, very similar values were obtained, although in the experiment shown in Fig. 2, pMT2-ECATΔ1 yielded less CAT′ polypeptide than the construct without the deletion. It is surprising that ECATΔ1 mRNA produces such large amounts of CAT′. We speculate that this is due to the adenovirus tripartite leader which may direct efficient translation regardless of the deletion of a portion of EMCV sequence. In contrast, the same deletion of EMCV 5′NTR in di- and tricistronic mRNA reduces the translational efficiency approximately 100-fold. This reduction is particularly noteworthy in pMT2-ECATΔ2-transfected cells, in which the EMCV 5′NTR carrying the deletion is preceded by the

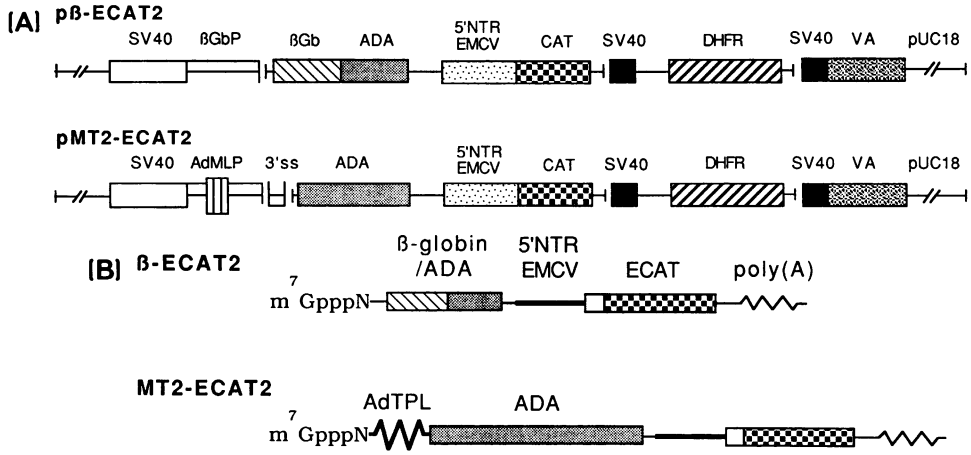

FIG. 5. Structure of dicistronic expression vectors and their mRNA transcripts. (A) The vector pβ-ECAT contains the promoter, the 5'NTR, and the N-terminal 122-amino-acid-coding sequence of the human β-globin gene as well as the C-terminal 103-amino-acid-coding sequence of the ADA gene, the 5'NTR of EMCV, the CAT gene, and other controlling elements as described in the legend to Fig. 1A. Plasmid pMT2-ECAT2 was described in the same legend. Figures are not drawn to scale. (B) Dicistronic mRNAs transcribed from pβ-ECAT2 and pMT2-ECAT2. The β-globin/ADA hybrid cistron is represented by hatched and shaded boxes. All other elements are represented as described for Fig. 1B.

ADA gene that was strongly expressed (Fig. 2A, lanes h and j).

Translation of dicistronic mRNAs in poliovirus-infected cells. Poliovirus infection results in the rapid inhibition of cellular, cap-dependent protein synthesis (10, 53). This is thought to be due to the modification of eIF-4F, a cellular protein complex that binds capped mRNAs (11, 61). We have studied the effect of poliovirus infection of COS-1 cells on translation of a dicistronic mRNA consisting (in the 5′ to 3′ direction) of the 5′NTR and the N-terminal coding sequence of human β-globin, the C-terminal coding sequence of ADA, the 5′NTR of EMCV, and the ECAT cistron (Fig. 5B, β-ECAT2). This mRNA is transcribed from vector pβ-ECAT2 (Fig. 5A). A segment of the β-globin gene was fused to a segment of the ADA gene such that the β-globin/ADA hybrid polypeptide (referred to as β-Globin′) could be immunoprecipitated with anti-ADA antibody.

COS-1 cells, which are derivatives of CV-1 monkey kidney cells and carry a poliovirus receptor (6), were transfected with pβ-ECAT2 or pMT2-ECAT2 and 48 h later were mock infected or infected with poliovirus. In a control experiment, we examined the extent of inhibition of host cellular protein synthesis in COS-1 cells in response to poliovirus infection (Fig. 6A, lanes a through c); inhibition was nearly complete at 3 h p.i. (lane b). Similar patterns of inhibition of host cellular protein synthesis by poliovirus infection were seen in vector-transfected COS-1 cells (Fig. 6A, lanes e, f, h, and i). Without immunoprecipitation, the expression of the β-Globin′ protein (indicated by an open triangle in Fig. 6A, lane d) and of the CAT′ protein (solid arrowhead in lane d) was barely visible in uninfected cells, although the ADA protein (open double triangle in lane g) was clearly apparent. No band migrating in the position of β-Globin′ was seen at 3 h p.i. (Fig. 6A, lane e). In contrast, a CAT′ band migrating slightly faster than poliovirus capsid protein VP3 was clearly visible 3 h p.i. (Fig. 6A, lane e). At 4 h p.i. this band was not seen unless the products were immunoprecipitated. We monitored CAT′ synthesis by using a monoclonal anti-CAT antibody and a secondary rabbit anti-mouse IgG antibody as described for the previous experiment, except that the cell extracts were preadsorbed with the secondary antibody (Fig. 6B). The β-Globin′- and ADA-related polypeptides were immunoprecipitated with anti-ADA antibodies (Fig. 6C). We conclude that synthesis of CAT′ was not inhibited as a result of poliovirus infection, regardless of whether the COS-1 cells were transfected with pβ-ECAT2 or with pMT2-ECAT2 (Fig. 6B, lanes e, f and h, i, respectively). By contrast, synthesis of β-Globin′ was not apparent at 3 h p.i. (Fig. 6C, lane d). Synthesis of ADA was unaffected in poliovirus-infected cells (Fig. 6C, lanes h and i; note that the ADA product migrated more slowly than poliovirus VP0 in this gel). It is apparent that the yields of CAT′ and ADA 4 h p.i. were reduced compared with those observed 3 h p.i. (Fig. 6A, lanes h and i; Fig. 6B, lanes h and i; Fig. 6C, lanes h and i). This is not surprising since at 4 h p.i. with 100 PFU of poliovirus per cell the nuclear transcripts originating from these vectors must compete with large numbers of poliovirus mRNA molecules for translation.

These results demonstrate that translation of the second cistron (ECAT) of β-ECAT2 occurs independently of translation of the first cistron (β-globin/ADA).

DISCUSSION

In vitro translation of synthetic mono- and dicistronic mRNAs containing a segment of the EMCV 5′NTR has previously led us to conclude that this viral genetic element contains an IRES capable of functioning independently of the 5′ end of the mRNAs (20). We have designed experiments to test such an IRES function in vivo, since translation in vitro may yield aberrant results.

We employed two different strategies. The first involved translation of polycistronic mRNAs whose leading cistron (in this instance, ADA) was always translated very efficiently due to the presence of the capped adenovirus tripartite leader. The second cistron was either a reporter gene (DHFR) preceded by a cellular 5′NTR or another reporter gene (ECAT) preceded by a picornaviral 5′NTR. In tricistronic mRNAs, the genetic units were arranged in the order ADA-DHFR-ECAT (Fig. 1B). We observed that ECAT controlled by the EMCV 5′NTR was always translated, regardless of its position in the mRNAs, whereas DHFR, translated from a gene in position 2, was not detected. These

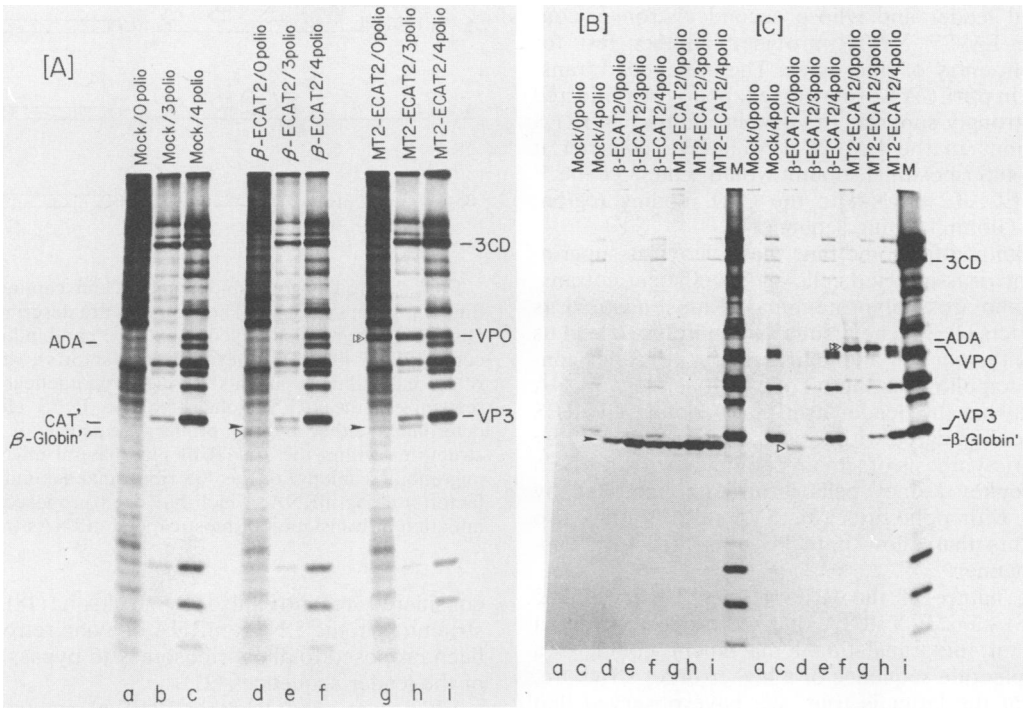

FIG. 6. Effect of poliovirus infection on translation of dicistronic mRNAs. (A) Total cell extracts from mock-transfected and dicistronic vector-transfected COS-1 cells. COS-1 cells transfected with the plasmids shown in Fig. 6A were infected with poliovirus at 48 h p.t. Cells were labeled with [^{35}S]methionine for 30 min at 0 (lanes a, d, and g), 3 (lanes b, e, and h), or 4 (lanes c, f, and i) h p.i. Labeled proteins were analyzed by SDS-PAGE. Lanes: a through c, mock-transfected cells; d through f, COS-1 cells transfected with pβ-ECAT2; g through i, COS-1 cells transfected with pMT2-ECAT2. The solid arrowhead and the open triangle in lane d indicate the CAT′ and β-Globin′ bands, respectively. The open double triangle in lane g indicates the ADA band. (B) Immunoprecipitation of polypeptides described in the legend to panel A with a monoclonal anti-CAT antibody. The labels and symbols are as described in the same legend. Note that nonspecific precipitation was greatly reduced by preadsorption of the COS-1 cell lysate with the secondary antibody (rabbit anti-mouse IgG antibody) before the primary antibody was added. (C) Immunoprecipitation of polypeptides described in the legend to panel A, using anti-ADA polyclonal antibodies. The coprecipitation of poliovirus capsid proteins, apparent in panels B and C, is a phenomenon which has been noted previously with other polyclonal antibodies (54). The β-Globin′ (a β-globin/ADA hybrid protein) was recognized by the polyclonal anti-ADA antibodies as indicated by the open triangle in lane c.

results suggest that ECAT translation independent of upstream sequences in di- and tricistronic mRNAs is due to the EMCV 5′NTR. It could be argued that ribosomal leaky scanning or a mechanism of reinitiation (28, 34, 45, 46) facilitated expression of the ECAT coding sequence in the second or third cistron. We do not believe this can account for our results, for the following reasons. First, we could not detect DHFR translation in pMT2-ADA-transfected cells to levels equaling that of ECAT in position 2 (note the absence of an upstream AUG in the 5′NTR of the DHFR-coding sequence). Similarly, ribosomal leaky scanning or reinitiation could hardly provide enough ribosomes for translation of the ECAT cistron in position 3. Second, an additional deletion in the EMCV 5′NTR of 225 nt reduced the translation of the ECAT in positions 2 and 3 up to 100-fold. This cannot be due to the absence of a ribosomal recovery sequence (31) since the remainder of the viral 5′NTR is 349 nt long. It is also highly unlikely that a stable secondary structure was formed in the MT2-ECATΔ2 and MT2-ECATΔ3 mRNAs which could have prevented resumption of scanning (26, 30, 48), because the MT2-ECATΔ1 mRNA which contains the same segment of the EMCV 5′NTR was translated as well as its undeleted counterpart. Moreover, a series of deletions in the EMCV 5′NTR did not restore the translational efficiency of dicistronic mRNAs in vitro (20). We therefore conclude that ribosomes (or 40S ribosomal subunits) attach to an IRES element independently of the 5′ end of the mRNA and that a deletion in the EMCV 5′NTR destroys the function of the IRES element. Results from in vitro experiments involving hybrid arrest of translation of EMCV RNA (57) can also be interpreted to indicate internal entry of ribosomes.

We do not suggest that ribosomal leaky scanning or reinitiation does not occur at all in the polycistronic mRNAs analyzed here. The very low CAT activity observed in cells transfected with pMT2-ECATΔ2 and pMT2-ECATΔ3 (Fig. 3) may be the result of ribosomal reinitiation. Alternatively, it could reflect residual function of the IRES element impaired by the deletion of nt 260 to 484 of the EMCV 5′NTR.

The second strategy to test the IRES function made use of the inhibition of host cellular protein synthesis in poliovirus-infected cells. Shutoff of cellular mRNA translation occurs soon after infection, although the mechanism by which it occurs is not fully understood. The available evidence suggests that inhibition is due to cleavage of a component (polypeptide p220) of the cellular cap-binding protein complex eIF-4F (10, 60). The cleavage of p220 is induced, but not directly effected, by poliovirus proteinase 2A (2, 32), although not all picornaviruses induce cleavage of p220 (37). Picornavirus mRNAs are uncapped, so that inactivation of eIF-4F would not affect picornaviral mRNA translation. It follows that the efficient translation of picornavirus mRNA may be due to its long 5′ leader. Analysis of expression of a dicistronic mRNA whose first cistron is controlled by a

cellular capped leader and whose second cistron is controlled by the EMCV 5'NTR provided another test for cap-independent entry of ribosomes. The unimpeded translation of CAT' in pβ-ECAT2-transfected, poliovirus-infected cells (Fig. 6) strongly supports the conclusion that an IRES element functions in the EMCV 5'NTR. We consider it unlikely that a 40S ribosomal subunit would scan from the 5' end of the β-ECAT2 mRNA to the CAT'-coding region, ignoring the β-Globin'-coding sequence.

It has been known for some time that poliovirus superinfection of adenovirus-infected cells allows efficient cotranslation of the poliovirus polyprotein and of most adenovirus late gene products (1, 3). The results shown in Fig. 6 lead us to suggest that the capped adenovirus tripartite leader confers resistance to poliovirus shutoff of cap-dependent mRNA translation and that this leader itself may contain an IRES element. These data agree with those published recently by Dolph et al. (8). Synthesis of simian parainfluenza virus SV5 proteins in monkey kidney cells is also not inhibited by superinfection with poliovirus (5). SV5 mRNA may also contain elements that allow it to be translated in a cap-independent manner.

What is the nature of the IRES element? The genetic element (IRES) of EMCV RNA that we propose to direct internal entry of the translational machinery could be a continuous nucleotide sequence or a higher-order structure. We believe that the latter is true. We have observed that various deletions between nt 260 and 800 of the EMCV 5'NTR destroy IRES function (S. K. Jang and E. Wimmer, unpublished results), an observation suggesting that distant RNA sequences contribute to the formation of an IRES. The presence in the 5'NTR of poliovirus of an IRES element with properties similar to those described here for EMCV RNA has been demonstrated recently by Pelletier and Sonenberg (49). Since the 5'NTRs of poliovirus and EMCV do not bear significant homology in primary sequence, higher-order structures of these mRNAs may be responsible for IRES function. Such a structure involving distant nucleotide sequences would explain very recent data by Trono et al. (64), who performed an extensive mutational analysis of poliovirus 5'NTR and concluded that "an RNA sequence of hundreds of nucleotides at the 5' end of poliovirus is involved in allowing viral protein synthesis." Secondary structures of the 5'NTRs of picornaviruses are currently being mapped (51a), but no common elements have emerged, and no relation between the proposed secondary structures and viral RNA function(s) has been established. The nature of the protein factor(s) and the state of the ribosome which both interact with the viral RNA structure are also unknown. Factors may attach to an IRES element upstream of the initiator AUG codon and direct ribosomal subunits to this region of the 5'NTR (Fig. 7, model 2). HeLa cell factors which correct aberrant translation of poliovirus mRNA in rabbit reticulocyte lysate (9, 51, 62) may serve as such. Moreover, the mRNAs of different genera of *Picornaviridae* may respond to distinct factors for their translation, a possibility supported by their different abilities to function as mRNAs in rabbit reticulocyte lysates (9, 51, 58, 62).

The transfer of the initiation complex to the initiator codon could be accomplished by scanning, a mechanism resembling the scanning by the ribosomal subunit in cap-dependent eucaryotic cellular protein synthesis (Fig. 7, model 1). Alternatively, the IRES element could involve noncontiguous segments of RNA, resulting in attachment of the translational machinery in the immediate vicinity of the initiator AUG codon (Fig. 7, model 3). Such a mechanism has been considered recently also by Jackson (18). Higher-order structures in the 5'NTR mRNA of avian retrovirus have also been proposed to allow ribosomes to bypass major portions of the leader sequences (21).

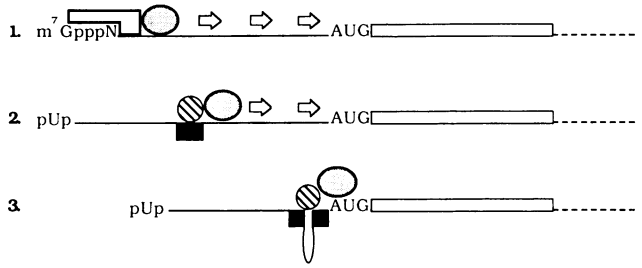

FIG. 7. Models for cap-dependent and cap-independent initiation of protein synthesis. These models are described in detail in the Discussion. Symbols: angled open box, cap-binding protein (CBP) complex (eIF-4F) and other initiation factor(s); shaded oval, a 40S ribosomal subunit; hatched circle, hypothetical factor(s) which recognizes, the IRES; solid box, the IRES element, which is determined either by the primary sequence or by higher-order structures within the 5'NTR of picornaviral mRNA; open arrow, migration (scanning) of the 40S ribosomal subunit and/or initiation factor(s) along mRNA, which has been proposed to occur during initiation of translation of most capped mRNAs (model 1 [25, 27]).

We do not know whether IRES elements are confined to specific viral mRNAs or whether they exist also in some cellular mRNA species. If the latter is true, studies on the function of IRES elements in picornaviruses may reveal new mechanisms of gene regulation at the level of translation.

ACKNOWLEDGMENTS

We thank Hans-Georg Kräusslich and Christopher U. T. Hellen for very useful suggestions and discussions and C. U. T. Hellen, H.-G. Kräusslich, and Bert L. Semler for critical reading of the manuscript. We are grateful to Patrick Hearing and Winship Herr for providing plasmids pSV2-CAT and pβe⁻, to Ann C. Palmenberg for the plasmid pE5-LVP0, to Rodney Kellems for the anti-ADA and anti-DHFR antisera, and to Nahum Sonenberg for the monoclonal anti-CAT antibody.

This work was supported in part by Public Health Service grants AI-15122 and CA-28146 to E. W. from the National Institutes of Health.

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