JOURNAL OF VIROLOGY, Nov. 1991, p. 6194–6204
0022-538X/91/116194-11$02.00/0

Vol. 65, No. 11

Translation of Poliovirus RNA: Role of an Essential *cis*-Acting Oligopyrimidine Element within the 5′ Nontranslated Region and Involvement of a Cellular 57-Kilodalton Protein

TATYANA V. PESTOVA,† CHRISTOPHER U. T. HELLEN,* AND ECKARD WIMMER

Department of Microbiology, State University of New York at Stony Brook, Stony Brook, New York 11794-8621

Received 4 April 1991/Accepted 23 July 1991

Translation of poliovirus RNA is initiated by cap-independent internal entry of ribosomes into the 5′ nontranslated region. This process is dependent on elements within the 5′ nontranslated region (the internal ribosomal entry site) and may involve novel translation factors. Systematic mutation of a conserved oligopyrimidine tract has revealed a *cis*-acting element that is essential for translation in vitro. The function of this element is related to its position relative to other *cis*-acting domains. This element is part of a more complex structure that interacts with several cellular factors, but changes in protein binding after mutation of this element were not detected in a UV cross-linking assay. A 57-kDa protein from the ribosomal salt wash fraction of HeLa cells was identified that binds upstream of the oligopyrimidine tract. Translation of poliovirus mRNA in vitro was strongly and specifically inhibited by competition with the p57-binding domain (nucleotides 260 to 488) of the 5′ nontranslated region of encephalomyocarditis virus, indicating a probable role for p57 in poliovirus translation. p57 is likely to be identical to the ribosome-associated factor that binds to and is necessary for the function of the internal ribosomal entry site of encephalomyocarditis virus RNA.

The genome of poliovirus type 1 (Mahoney) consists of a single-stranded, ca. 7,500-nucleotide (nt) RNA molecule of positive polarity (27). It resembles eukaryotic mRNA molecules in being monocistronic and encodes a single 247-kDa polyprotein that is cleaved at specific sites to generate poliovirus proteins (for a review, see reference 17). However, in contrast to all other known eukaryotic cytoplasmic mRNAs, the RNA of poliovirus and of other picornaviruses is not linked at its 5′ end by the cap structure m^7GpppX (where X is any nucleotide). Instead, all picornavirus genomes are covalently linked at their 5′ termini to a small polypeptide, called VPg, that is removed from viral RNA. As a result, the 5′ end of polysomal poliovirus RNA is pUp (see reference 76 for references). The cap structure of cellular mRNA serves as a binding site for a cap-binding protein complex, which is known to be required for the initiation of eukaryotic protein synthesis (57), so that poliovirus RNA must in some way circumvent the requirement for a cap structure for its translation. Indeed, translation of poliovirus mRNA in vivo occurs under conditions in which eIF-4F (cap-binding protein complex) is inactivated (67).

Initiation of translation of eukaryotic mRNAs occurs predominantly at the most 5′-proximal AUG, although the absence of a favorable context [CC(A/G)CCAUGG] may explain exceptional instances when initiation occurs at a second or subsequent AUG (29). The mechanism by which the initiation complex moves from the 5′ terminus to the correct AUG start site is obscure, although stable secondary structures in the intervening 5′ nontranslated region (5′-NTR) reduce the efficiency of initiation (28, 52). Initiation of translation of RNA of poliovirus type 1 (Mahoney) is thus exceptional in that it occurs at the ninth AUG triplet of a 5′-NTR that, in addition to being uncapped, is very long (742 nt) (13, 27) and contains several stable secondary-structure elements (35, 36, 56, 66). Longstanding suspicions that initiation of poliovirus translation occurs by ribosomal binding to the 5′-NTR independently of the 5′ terminus have only recently been confirmed (53). Similar experiments indicated that the RNA of encephalomyocarditis virus (EMCV; a picornavirus of the cardiovirus genus) contains a functionally related genetic element that has been called the internal ribosomal entry site (IRES) (23, 24). Structural and experimental considerations suggest that the 5′-NTRs of members of the other two genera of picornaviruses (rhinoviruses and aphthoviruses, e.g., foot-and-mouth disease virus [FMDV]) also contain IRES elements (4, 32, 55, 56).

A number of possible mechanisms for the internal entry of ribosomes into poliovirus RNA have been suggested (18, 22, 25, 68), but in fact most details of how this occurs have not yet been elucidated.

The 5′ boundary of the poliovirus IRES has not been mapped precisely, although deletion of the first 95 nt of the 5′-NTR has been found to have no significant effect on cap-independent internal ribosomal binding (50). Although the sequences, and thus presumably the secondary structures, of the 5′-NTRs of different serotypes and isolates of poliovirus (and indeed of all rhino- and enteroviruses) are generally very similar, considerable variation occurs within a 100-nt segment preceding the initiation codon (74). This segment is completely absent in rhinoviruses and is nonessential in poliovirus; deletion of nt 600 to 726 did not alter the phenotypic properties of poliovirus type 1 (Sabin) in tissue culture (31). However, deletion of nt 564 to 726 resulted in virus progeny with a small-plaque phenotype (19, 31), and further downstream deletion to nt 561 abolished viral infectivity (19). These experiments suggest that the 3′ border of the sequence necessary for efficient internal entry in HeLa cell extracts extends until at least nt 563. This boundary falls within a pyrimidine-rich (particularly uridine-rich) tract that is highly conserved in entero- and rhinoviruses (Table 1). A similar oligopyrimidine tract occurs near the initiating AUGs of FMDV (6, 61), hepatitis A virus (48), and all cardiovi-

* Corresponding author.
† Permanent address: A. N. Belozersky Laboratory of Molecular Biology, Moscow State University, 119899 Moscow, USSR.

TABLE 1. Sequence variation in an oligopyrimidine tract conserved in 5′-NTRs of all enteroviruses and rhinoviruses[a]

Virus and serotype or strain	Nucleotide sequence																					
Polioviruses																						
Serotype 1 (Mahoney)	554	G	U	G	U	U	U	C	C	U	U	U	U	A	U	U	U	U	A	U	U	573
Serotype 1 (Sabin)	556	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	575
Serotype 2 (Sabin)	561	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	U	A	·	580
Serotype 2 (Lansing)	557	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	U	A	C	576
Serotype 2 (W2)	560	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	U	A	C	579
Serotype 3 (Sabin)	561	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	G	A	580
Serotype 3 (Leon)	559	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	U	G	A	578
Serotype 3 (Finland)	559	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	C	A	·	578
Echoviruses																						
25		·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	C	·	U	A	·	
34		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	U	·	A	
Enterovirus 70	559	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	U	A	·	578
Coxsackieviruses (type B)																						
B1	559	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	C	C	U	A	·	578
B3	558	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	C	C	U	A	·	577
B4	561	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	U	A	C	580
Swine vesicular disease viruses																						
H/3 1976	559	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	U	A	C	578
UKG/27/72	559	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	C	·	U	A	·	578
Bovine enterovirus	638	·	·	·	·	·	·	·	·	·	·	A	·	U	A	·	·	·	U	A	·	657
Coxsackieviruses (type A)																						
A9	561	·	·	·	·	·	·	·	U	·	·	·	·	·	·	·	C	C	U	A	·	580
A16		·	·	·	·	·	·	·	·	·	A	·	·	U	C	C	·	·	U	A	C	
A21	554	·	·	·	·	·	·	·	·	C	·	·	·	·	·	A	·	·	C	A	·	573
Rhinoviruses																						
1B	557	·	·	·	·	·	·	·	A	C	·	·	·	U	·	C	C	·	U	·	A	565
2	546	·	·	·	·	·	·	·	A	C	·	·	·	U	·	C	C	·	U	·	A	565
9	547	·	·	·	·	·	·	·	U	C	·	·	·	U	·	C	C	·	U	·	·	566
14	562	·	·	·	·	·	·	·	U	C	A	·	·	U	·	·	C	·	U	C	A	581
85	548	·	·	·	·	·	·	·	A	C	·	·	·	U	·	A	C	C	U	·	·	567
89	554	·	·	·	·	·	·	·	·	·	G	·	·	U	·	·	C	·	U	·	·	573
Mutant constructions																						
wt	554	G	U	G	U	U	U	C	C	U	U	U	U	A	U	U	U	U	A	U	U	573
1	554	·	·	·	G	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	573
2	554	·	·	·	·	·	A	A	·	·	·	·	·	·	·	·	·	·	·	·	·	573
3	554	·	·	·	·	·	·	G	G	·	·	·	·	·	·	·	·	·	·	·	·	573
4	554	·	·	·	·	·	·	·	·	G	G	·	·	·	·	·	·	·	·	·	·	573
5	554	·	·	·	·	·	·	·	·	·	·	C	C	·	·	·	·	·	·	·	·	573
6	554	·	·	·	·	·	·	·	·	·	·	G	G	·	·	G	G	·	·	·	·	573
7	554	·	·	·	G	A	·	·	·	·	·	C	C	·	·	G	G	·	·	·	·	573
8	554	·	·	·	·	·	·	·	·	·	·	C	C	·	·	G	G	·	·	·	·	573
9	554	·	·	·	·	·	·	·	·	·	·	·	·	U	·	C	C	·	U	·	·	573
10	554	·	·	·	·	·	·	G	G	·	·	·	C	C	·	·	·	·	·	·	·	573

[a] Aligned nucleotide sequences of the oligopyrimidine tracts of 12 enteroviruses and 6 rhinoviruses. All but four of the nucleotide sequences used in this alignment are discussed in a recent review (69); for the nucleotide sequence of swine vesicular disease virus (strain UKG/27/72), see reference 63; for the nucleotide sequences of echoviruses 25 and 34 and of coxsackievirus A16, see reference 10. The numbers of nucleotides that occur at the 5′ and 3′ borders of the sequences under consideration are given when they are known. Nucleotides that are different from those of poliovirus type 1 (Mahoney) are shown, and nucleotides that are the same are indicated by a dot. Also shown are the nucleotide sequences of mutations generated by site-directed mutagenesis. Nucleotides that are different from the parental (wt) sequence are shown, and nucleotides that are the same are indicated by a dot. Mutations are referred to by numbers, and these are used in the text to refer to variants of pPV1(M)5′-P1 that carry these changes from the wt sequence.

ruses, including EMCV (47). Deletion analysis suggests that this motif is close to the 3′ border of the EMCV IRES (26), and mutational analysis demonstrated that it constitutes an essential domain for FMDV translation (32). However, the role of this conserved tract in rhinoviruses and enteroviruses (such as poliovirus) has not yet been established.

Several lines of evidence indicate that the cap-independent mechanism of poliovirus RNA translation may involve novel *trans*-acting cellular factors. Translation of poliovirus mRNA is relatively restricted in human blood and neural cells (34, 39) but occurs efficiently in HeLa cells. It is similarly efficient in cell extracts of HeLa cells and Krebs-2 cells (49, 72) but is restricted in rabbit reticulocyte lysate (RRL [14]), although the translational deficiency in RRL can

be relieved by supplementation with a crude mixture of translation initiation factors (ribosomal salt wash) from nucleated (e.g., HeLa or Krebs-2) cells (9, 14). The low translation efficiency can be attributed to sequences in the 5′-NTR of poliovirus RNA, since deletion of much of the 5′-NTR dramatically increased translation efficiency in RRL (45). Interaction with tissue-specific *trans*-acting factors is likely to be an important determinant of viral pathogenesis, and there is indeed considerable evidence to suggest that the 5′-NTR of poliovirus is involved in determining the level of viral neurovirulence (for reviews, see references 1, 3, and 46). The most likely reason is that attenuating mutations impair the ability of RNA to initiate translation (70, 72). Moreover, the expression of attenuating mutations is tissue specific in vivo (2, 34) and in vitro (73).

The technique of UV cross-linking [^{32}P]UTP-labeled RNA transcripts to cellular RNA-binding proteins has recently been used to identify a protein, p52, that bound specifically to the nt 559 to 624 segment of poliovirus type 2 (Lansing) (42). The interaction appears to involve a domain that, although important for translation (7, 51), is not essential (19, 31). It is therefore likely that additional factors interact with other regions of the 5′-NTR, probably including the domain that contains tissue-specific determinants of the neurovirulent phenotype. The UV cross-linking technique has also recently been used to detect novel cellular proteins, variously described as p57 and p58, that interact specifically with the 5′-NTRs of EMCV (8, 26) and FMDV (40). Specific binding of p57 to the IRES of EMCV was found to correlate with efficient translation in vitro (26).

To gain a better understanding of the mechanism of translation initiation of poliovirus mRNA, we have analyzed the effects of systematic mutation within the oligopyrimidine tract of poliovirus type 1 (Mahoney) mRNA on its translation in three in vitro systems. As a result, we have identified a *cis*-acting element within this tract that is essential for translation in vitro. This element is part of a more complex structure that interacts with several cellular factors; we have confirmed binding of p52 to the previously identified site (42) and have additionally demonstrated binding of this protein to noncontiguous upstream and downstream sequences. We have also shown that p57 binds to the 5′-NTR and that it appears to be an important cofactor for the translation of poliovirus RNA in vitro. The possible roles of these *trans*-acting protein factors and the essential *cis*-acting RNA element are discussed in light of current models for initiation of picornavirus translation by internal entry of ribosomes (22, 25, 68).

MATERIALS AND METHODS

Genetic engineering of DNA. Restriction enzymes and DNA-modifying enzymes were purchased either from New England BioLabs or Bethesda Research Laboratories, Inc. *Taq* polymerase and reagents for the polymerase chain reaction (PCR) were purchased from Perkin Elmer Cetus. Mutagenic oligonucleotides and primers for sequencing, transcription, and PCR amplification were synthesized on an Applied Biosystems apparatus. DNA manipulations were done by standard procedures (60).

Bacterial strains and plasmid construction. The plasmid pBS$^+$VP0 (30) was used to introduce site-directed mutations within the 5′-NTR of poliovirus type 1 (Mahoney). This vector carries a *Kpn*I-*Nru*I fragment (nt 70 to 1174 of the poliovirus cDNA) cloned between the *Kpn*I and *Hin*cII sites in the polylinker region of pBS(KS$^+$) (Stratagene), which contains an M13 origin as well as a ColE1 origin and a *bla* gene for amplification in *Escherichia coli*. Single-stranded DNA substituted with uracil was prepared by passage through *E. coli* BW313 (*dut ung* mutant) by the method of Kunkel (33). Synthetic deoxyoligonucleotides were annealed to the DNA, and second-strand synthesis and transformation into *E. coli* C600 were done as described previously (33). Mutant sequences were identified by sequence analysis with the primer 5′-CCGGAGCTCTCCTCCGGCCCCTGAATG-3′ (the 3′ portion of which is equivalent to nt 443 to 460 of poliovirus type 1 [Mahoney] [27]) and the dideoxynucleotide chain termination method (62).

To investigate the effect of mutations within the 5′-NTR of poliovirus on its ability to direct translation in vitro, we cloned mutated sequences into a transcription vector, pPV1(M)5′-P1, that contains most of the 5′-NTR of poliovirus type 1 (Mahoney) (nt 70 to 742) and the entire P1 capsid protein precursor coding region (nt 743 to 3380), followed by an opal (UGA) termination codon. This fragment of the poliovirus genome was placed under the transcriptional control of a T3 polymerase promoter. pPV1(M)5′-P1 and derivatives thereof were created by ligating a *Ban*II-*Pvu*II fragment from wild-type (wt) and mutated versions of pBS$^+$VP0 (which contains a portion of the 5′-NTR from nt 70 to the initiation codon) to a *Ban*II-*Pvu*II fragment derived from pMN22 (45), which contains the 5′-NTR from nt 670 to the initiation codon, and the entire P1 coding region. Mutations in the oligopyrimidine tract were verified by sequence analysis of cDNA in these plasmids corresponding to nt 70 to 670 of the poliovirus 5′-NTR.

Oligoribonucleotide synthesis with T7 polymerase and DNA templates synthesized by PCR. In order to analyze possible interactions between cellular proteins and the wild-type and mutated versions of the oligopyrimidine tract, we have developed a variation of a previously described technique (43) to synthesize [^{32}P]UTP-labeled RNA transcripts for use in UV cross-linking assays (see Fig. 3A). The deoxyoligonucleotides 5′-GGCCAATCCAATTGCAATTCGC-3′ and 5′-TAATACGACTCACTATAGGGGCTTTGGGTGTCCGTG 3′ were used to copy the cDNA sequence of poliovirus type 1 (Mahoney) from nt 544 to 631 by PCR. The first 17 nt of the second, larger deoxyoligonucleotide primer correspond to the bacteriophage T7 ϕ10 promoter (16); three G residues were included between the poliovirus-specific sequence to increase the efficiency of transcription by T7 polymerase (43). PCR was done with a Perkin Elmer Cetus DNA thermal cycler as described before (20), except that dissociation of DNA duplexes at 94°C was limited to 45 s in duration, annealing was done at 50°C for 45 s, and the elongation stage was omitted. PCR products were purified after electrophoresis in a 2% low-melting-point agarose gel and were used to synthesize [^{32}P]UTP-labelled RNA transcripts with T7 polymerase under standard conditions. RNA transcripts were purified after electrophoresis in a 5% polyacrylamide gel.

UV cross-linking of RNAs. The UV cross-linking reaction was performed essentially as described by Meerovitch et al. (42), with some modifications. Gel-purified RNA probes (10^5 to 10^6 cpm) that had been labeled with [^{32}P]UTP during transcription with T7 polymerase, were incubated for 10 min at 30°C in a HeLa cell extract (25 μl total volume) that had been optimized for translation of poliovirus RNA (see above). Samples were irradiated with UV light on ice for 30 min with a UV-Stratalinker (Stratagene). Unbound RNAs were digested with 20 μg of RNase A, 20 U of RNase T_1, and 1 U of cobra venom nuclease by incubating at 37°C for 30 min. Samples were analyzed by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. Ribosomal salt wash from HeLa S3 cells was prepared by the method described by Schreier and Staehelin (63).

We generated [^{32}P]UTP-labeled RNA probes corresponding to segments of the 5′-NTR of poliovirus type 1 (Mahoney) by transcribing fragments of pPV1(M)5′-P1, pT7XL (a derivative of pT7PV1-5 [75]), pMN22 (45), and derivatives thereof. These plasmids were digested with the restriction enzymes *Bam*HI, *Bal*I (or *Msc*I), and *Hgi*AI, which have sites at nt 220 and 670, nt 627, and nt 747 of poliovirus, respectively.

Two specific probes were used to bind cellular proteins that have previously been described as binding to picornavirus IRES elements. Thus, as a positive control for the binding of p52, a [^{32}P]UTP-labeled RNA transcript corresponding to nt 559 to 624 of poliovirus type 2 (Lansing) was synthesized as described before (42). As a positive control for the binding of p57, a [^{32}P]UTP-labeled RNA transcript corresponding to nt 260 to 488 of EMCV (47), and therefore containing stem-loop E, which has been shown to bind p57 (26), was transcribed with T7 polymerase, using plasmid pBS-ECAT (23) that had been linearized by digestion with *Hin*dIII.

In competition experiments, 1 to 2.5 μg of unlabeled rabbit rRNA (Sigma) and 0.5 μg of unlabeled poliovirus transcript (corresponding to nt 559 to 624) or 0.5 μg of unlabeled EMCV RNA transcript (corresponding to nt 260 to 488) were added to aliquots of HeLa cell extract simultaneously with specific [^{32}P]UTP-labeled poliovirus RNA probes.

The molecular masses of proteins other than p52 and p57 were estimated relative to those of ^{14}C-labeled protein molecular mass markers (Amersham International), which included ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase B (97.4 kDa), and myosin (200 kDa).

Transcription and translation in vitro. Plasmid pPV1(M)5′-P1 (wild type and derivatives thereof containing substitutions in the pyrimidine-rich tract) was linearized by digestion with *Eco*RI and transcribed in vitro with T3 RNA polymerase, using conditions described by the manufacturer. RNA transcripts were purified by using Nuctrap push columns (Stratagene) exactly as described by the manufacturer, and their concentrations were determined by spectrophotometry. The various purified RNA transcripts were also compared in an ethidium bromide-stained agarose gel to confirm that equal concentrations were used in translation. Synthetic mRNA transcripts were translated in RRL (Promega Biotec), HeLa cell extract, or Krebs-2 cell extract, as appropriate, in the presence of [^{35}S]methionine for 60 min at 30°C. Preparation of cell extracts from HeLa S3 cells and translation in these extracts were done as described before (57) with minor modifications (38). Cell extracts of Krebs-2 cells were prepared and used in translation experiments as described previously (71, 72). The concentration of mRNA included per 25-μl aliquot of cell lysate is indicated in the text. The products resulting from in vitro translation were resolved by SDS-PAGE (45). Translation efficiencies were quantitated by scanning X-ray films with an LKB laser densitometer. A [^{35}S]methionine-labeled cell lysate was prepared from HeLa cells that had been infected with poliovirus type 1 (Mahoney), as described by Lee and Wimmer (37), for use as a marker.

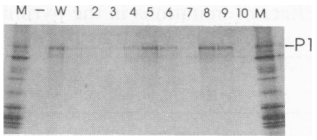

FIG. 1. Translation of pPV1(M)5′-P1 poliovirus mutant RNAs in HeLa cell lysate. RNA derivatives (Table 1) were translated for 60 min at 30°C, at 1.5 μg/25 μl, and aliquots of the translation assay mixture were separated by electrophoresis on a 10 to 20% polyacrylamide gradient gel. Lanes are labeled to indicate the mutant RNA (as defined in Table 1) used to program translation. Lane W corresponds to a lysate programmed with wt RNA, and lane — corresponds to lysate incubated without exogenous mRNA. Lanes M correspond to a lysate of poliovirus-infected HeLa cells labeled in vivo with [^{35}S]methionine. The P1 translation product (97 kDa) is indicated.

RESULTS

Generation of mutants. In order to determine the influence of a conserved oligopyrimidine tract (nt 557 to 573) on the ability of the 5′-NTR of poliovirus type 1 (Mahoney) to direct translation in vitro, a vector [pPV1(M)5′-P1] was constructed that contains a segment of the poliovirus genome consisting of nt 70 to 742 of the 5′-NTR and the entire P1 (structural protein precursor) coding region (nt 743 to 3380) under the transcriptional control of a T3 polymerase promoter. The first 70 nt of the 5′-NTR were omitted from our constructs to simplify cloning and because they are not important for cap-independent internal ribosomal binding (50). The P1 capsid precursor naturally occurs immediately downstream of the 5′-NTR, and we used it rather than a heterologous coding region as a reporter gene to assay the efficiency of translation because we considered it possible that the untranslated sequence and protein-coding regions interact in a manner that defines the normal translational capacity of poliovirus RNA, in a manner similar to that suggested for yellow fever virus RNA (59). In order to avoid analysis of the several translation products generated by proteolytic processing of a larger precursor, we constructed plasmids so that a termination codon occurred immediately after the region encoding the P1 precursor.

Substitutions within the oligopyrimidine tract were made by site-directed mutagenesis with synthetic oligodeoxynucleotide primers. Mutations, shown in Table 1, were identified in derivatives of pBS$^+$VP0 by nucleotide sequence analysis and subsequently verified in the same way after construction of the various pPV1(M)5′-P1 variants.

Translation of pPV1(M)5′-P1 mutant RNAs in an extract from HeLa cells. A lysate of HeLa cells that had been experimentally optimized for translation of wt poliovirus mRNA by addition of 90 mM K^+ and 1.7 mM Mg^{2+} was used to assay the efficiency of translation of transcripts of the various pPV1(M)5′-P1 variants.

The effect of mutations in the pyrimidine-rich tract (Table 1) on the efficiency of translation of the P1 capsid protein precursor is shown in Fig. 1. The differences in translation efficiencies that are easily discernible from the intensities of the bands were quantitated by scanning laser densitometry and are summarized in Table 2. Substitutions within the 3′ half of the pyrimidine-rich tract (nt 566 to 573; mutants 5, 6, 8, and 9) had little or no effect, whereas all substitutions within the 5′ half (nt 559 to 565; mutants 1, 2, 3, 4, 7, and 10) greatly reduced the efficiency of translation of the P1 capsid protein precursor. Thus, translation of mutant 7 (which contains substitutions combining those introduced in mu-

TABLE 2. Effect of mutations in the pyrimidine-rich tract on efficiency of translation[a]

5'-NTR mutant RNA	Efficiency (% of wt)
wt	100
1	16
2	11
3	9
4	43
5	96
6	70
7	8
8	104
9	88
10	4

[a] Efficiency of translation of the P1 capsid protein precursor directed by various mutant RNAs (see Table 1) in a HeLa cell lysate (as described in the legend to Fig. 1) was quantitated by scanning X-ray films with an LKB laser densitometer and is described as a percentage of the efficiency of translation of wt RNA.

tants 1 and 8) is similar to translation of mutant 1 rather than mutant 8 (Fig. 1, lanes 1, 7, and 8). The essential core of this domain comprises nt 561 to 563 (UCC), since substitution of these residues (mutants 2, 3, and 10) resulted in the greatest reduction in efficiency of translation (Fig. 1, lanes 2, 3, and 10). This UCC domain and flanking sequences have essentially been restored in a position 4 nt further away from the 5' terminus in mutant 10, which otherwise resembles mutant 3 in containing substitutions at nt 562 and 563: wt, GUGUUUCCUUUUAUUUUAUUG; mutant 10, GUGUUUGGUUUCCUUUUAUUG. However, this change did not increase the efficiency of translation of mutant 10 over that of mutant 3 (compare lanes 3 and 10 of Fig. 1). It is therefore likely that the function of the essential part of the oligopyrimidine tract is related to its position relative to those of other *cis*-acting domains within the IRES, such as structural elements or protein-binding sites.

Translation of pPV1(M)5'-P1 mutant RNAs in extracts from Krebs-2 cells and in RRL. A lysate of Krebs-2 cells that had been optimized for translation of wt poliovirus mRNA (by addition of 90 mM K^+ and 1.7 mM Mg^{2+}) was used to assay the efficiency of translation of transcripts of the various pPV1(M)5'-P1 variants.

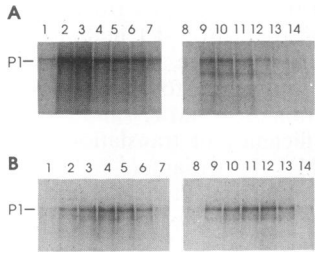

FIG. 2. Effect of RNA concentration on translation of pPV1(M)5'-P1 RNAs in HeLa and Krebs-2 cell lysates. Poliovirus RNAs were transcribed from wt pPV1(M)5'-P1 (lanes 1 to 7) or from mutant 3 (lanes 8 to 14). RNAs were translated in cell extracts of (A) HeLa S3 cells or (B) Krebs-2 cells for 60 min at 30°C at 4 μg (lanes 1 and 8), 2 μg (lanes 2 and 9), 1.5 μg (lanes 3 and 10), 1 μg (lanes 4 and 11), 0.5 μg (lanes 5 and 12), 0.3 μg (lanes 6 and 13), or 0.15 μg (lanes 7 and 14) per 25 μl. Aliquots of translation assay mixture were separated by electrophoresis on 10 to 20% polyacrylamide gels. The P1 translation product is indicated.

In contrast to translation in HeLa cell lysates, transcripts derived from variants of pPV1(M)5'-P1 were all translated with approximately equal efficiency in a Krebs-2 cell lysate (Fig. 2) and RRL (data not shown). This tissue-specific difference in sensitivity to mutation of the critical residues UCC (nt 561 to 563) is clearly illustrated in Fig. 2, which shows a comparison between the efficiency of translation of wt RNA and RNA derived from mutant 3 (which is the least efficiently translated of those shown in Fig. 1) over a range of RNA concentrations in HeLa and Krebs-2 cell lysates. Whereas the mutant RNA was indeed translated with lower efficiency than wt RNA in several independently prepared batches of HeLa cell lysate (compare Fig. 2A, lanes 1 through 7 [wt RNA] with lanes 8 through 14 [mutant RNA]), the two RNA species were translated with approximately equal efficiency at all RNA concentrations in a Krebs-2 cell lysate (compare Fig. 2B, lanes 1 through 7 [wt RNA] with lanes 8 through 14 [mutant RNA]). The unusual dose-response curve, whereby a higher mRNA concentration results in reduced synthesis of P1, is typical of poliovirus (21, 24, 54, 65).

Interaction of p52 with wt and mutated poliovirus 5'-NTRs. A novel protein (p52) has recently been described that can be specifically cross-linked to nt 559 to 624 of the poliovirus 5'-NTR (42), a segment which includes the oligopyrimidine tract. Binding of p52 to the 5'-NTR of poliovirus can apparently stimulate its translation in vitro (68). The pyrimidine-rich tract constitutes part of the probe originally used to detect p52, and we therefore anticipated that substitution in it might affect binding of this protein. To investigate this possibility by UV cross-linking techniques, we synthesized two sets of RNA probes. One of these corresponded to nt 70 to 627 and therefore included most of the poliovirus 5'-NTR. The second probe corresponded to nt 542 to 629 and was thus 22 nt longer than the probe used by Meerovitch et al. (42).

The binding of HeLa cell proteins to the nt 542 to 629 probe is shown first (Fig. 3B). The proteins that were detected included one that comigrated with p52 (lanes 1 and 2 of Fig. 3B). There was no difference between the amounts of p52 or any of the other proteins that bound to transcripts corresponding to the wt sequence and to the sequence of mutant 3. Interestingly, in our hands, the poliovirus type 1 (Mahoney) nt 542 to 629 probe bound considerably more proteins than the slightly smaller poliovirus type 2 (Lansing) nt 559 to 624 probe (Fig. 3B, compare lanes 2 and 3).

We next considered the possibility that substitution in the oligopyrimidine tract affected translation as a result of more complex changes in protein binding that involved the complete IRES. We found that proteins with a broad range of molecular masses (36 to 110 kDa) were UV cross-linked to [^{32}P]UTP-labeled RNA transcripts corresponding to nt 70 to 627 of the poliovirus type 1 (Mahoney) 5'-NTR (Fig. 4). There was no difference between the proteins that bound to wt and mutated transcripts (compare lane c of Fig. 4 with lanes d through h). We conclude that p52 binding is not affected by mutations in the oligopyrimidine tract.

We noted that the p52 that bound to these transcripts appeared as a broad band whose mobility was in part slightly greater than that of the protein described by Meerovitch et al. (42). By using a longer 10 to 20% polyacrylamide gradient gel and by extending the duration of electrophoresis, we were able to resolve this broad band into two components, one of which comigrated with the p52 species that bound to nt 559 to 624 (Fig. 5). The second component was 50 kDa and is therefore referred to as p50.

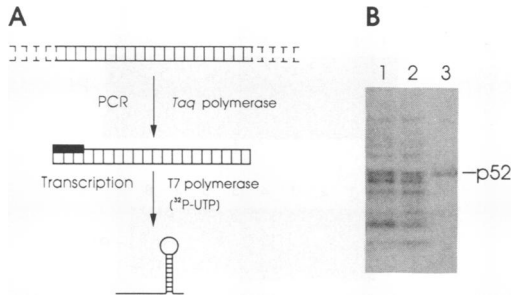

FIG. 3. Effect of mutations within the oligopyrimidine tract (nt 557 to 573) of poliovirus on binding of cellular proteins to the 5′-NTR: analysis of the interaction between proteins from HeLa S3 cell extracts and [^{32}P]UTP-labeled RNA transcripts corresponding to nt 542 to 629 by a UV cross-linking assay. (A) Diagram of the technique used to generate a set of poliovirus-specific transcripts, derived from wt and mutated derivatives of pPV1(M)5′-P1, that contain mutations within the oligopyrimidine tract. A segment of the poliovirus 5′-NTR (corresponding to nt 542 to 629) was amplified from pPV1(M)5′-P1 by PCR, using a pair of primers of which one included the bacteriophage T7 ϕ10 promoter sequence (represented by a black rectangle). Transcription was subsequently done with purified PCR products, [^{32}P]UTP, and T7 polymerase. The secondary structure of the transcript may include a stable and evolutionarily conserved stem-loop structure and an extended single-stranded region containing the oligopyrimidine tract. (B) UV cross-linking of cellular proteins to [^{32}P]UTP-labeled RNA transcripts (nt 542 to 629) from PCR products derived from wt pPV1(M)5′-P1 (lane 2) and from mutant 3 (lane 1). p52 bound to nt 559 to 624 of poliovirus type 2 (Lansing) is shown in lane 3. Proteins were separated by electrophoresis in a 10 to 20% polyacrylamide gradient gel after RNase digestion.

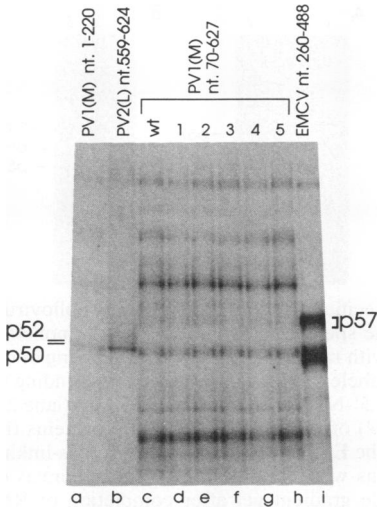

FIG. 4. Effect of mutations within the oligopyrimidine tract (nt 557 to 573) of poliovirus on binding of cellular proteins to the 5′-NTR: analysis of the interaction between proteins from HeLa S3 cell extracts and [^{32}P]UTP-labeled RNA transcripts corresponding to nt 70 to 627 by a UV cross-linking assay. UV cross-linking of cellular proteins to [^{32}P]UTP-labeled RNA transcripts corresponding to nt 1 to 220 (lane a), nt 70 to 627 of poliovirus type 1 (Mahoney) (lanes c to h), nt 559 to 624 of poliovirus type 2 (Lansing) (lane b), or nt 260 to 488 of EMCV (lane i). Transcripts were derived from pT7XL (lane 1), wt pPV1(M)5′-P1 (lane 3), mutant 1 (lane d), mutant 2 (lane e), mutant 3 (lane f), mutant 4 (lane g), or mutant 5 (lane h) and from pBS-ECAT (lane i). Proteins were separated by electrophoresis in a 10 to 20% polyacrylamide gradient gel after RNase digestion. p57, p52, and p50 are indicated.

We investigated the interaction of these proteins with the 5′-NTR of poliovirus by separating the S10 fraction of HeLa cells into ribosomal salt wash and postribosomal fractions and by transcribing a set of probes corresponding to different segments of the 5′-NTR. Both proteins could be detected in the postribosomal fraction and were present in much lower amounts in the ribosomal salt wash (Fig. 5, lanes 5 to 7, and data not shown). We were able to detect binding of p52 to the probe corresponding to nt 220 to 627 (which encompasses the probe used by Meerovitch et al. [42]; Fig. 5, lane 4), as well as to probes corresponding to nt 1 to 220 (Fig. 4, lane 1; Fig. 5, lane 1) and to nt 670 to 747 (Fig. 5, lane 2). p50 bound weakly to the nt 1 to 220 probe and strongly to the nt 220 to 627 probe, but it did not bind to the nt 670 to 747 probe (Fig. 5, lanes 1, 2, and 4). Binding of p52 to the poliovirus 5′-NTR was reduced by inclusion of unlabeled poliovirus type 2 (Lansing) nt 559 to 624 probe in the preincubation mixture, whereas binding of p50 was not affected (data not shown). We therefore conclude that p52 and p50 are distinct and probably unrelated proteins and that p50 binds to a region upstream of nt 559.

Interaction of p57 with the poliovirus 5′-NTR. As described above, a broad range of proteins were UV cross-linked to a probe corresponding to nt 70 to 627 of the poliovirus 5′-NTR (Fig. 4). To investigate the specificity of their binding, we performed competition experiments by adding different concentrations of rabbit rRNA to aliquots of HeLa S10 extract simultaneously with this specific probe. Binding of all proteins except for one of 57 kDa (which we shall refer to as p57) was progressively reduced following addition of increasing concentrations of competitor (Fig. 6A, lanes 2 to 4). Some of these proteins resemble those that bound nonspecifically to the 5′-NTR of EMCV (26). Similar proteins are components of eukaryotic cytoplasmic and polysomal messenger ribonucleoprotein particles (for reviews, see references 12 and 15).

The p57 detected in these experiments comigrated with p57 (26) that bound to nt 260 to 488 of EMCV (Fig. 6A). The identity of these proteins was confirmed by competition experiments. Binding of p57 to the poliovirus 5′-NTR was virtually abolished by inclusion of 0.3 μg of unlabeled EMCV nt 260 to 488 RNA probe in the preincubation mixture (Fig. 6B, lane 2). However, it was unaffected by addition of 0.3 μg of unlabeled poliovirus-specific RNA, corresponding to nt 559 to 624 (Fig. 6B, lane 3). The

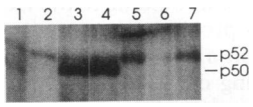

FIG. 5. Binding of p50 and p52 to the 5′-NTR of poliovirus. UV cross-linking of cellular proteins bound to [^{32}P]UTP-labeled poliovirus RNA transcripts corresponding to nt 1 to 220 (lane 1), nt 670 to 747 (lane 2), nt 1 to 627 (lane 3), or nt 220 to 627 (lane 4) of poliovirus type 1 (Mahoney) and to nt 559 to 624 of poliovirus type 2 (Lansing) (lanes 5, 6, and 7). HeLa cellular proteins were derived from a crude cytoplasmic S10 fraction (lanes 1 to 5), from the ribosomal salt wash fraction (lane 6), or from the postribosomal fraction (lane 7). Proteins were separated by electrophoresis on a 10 to 20% polyacrylamide gradient gel after completion of RNase digestion. p52 and p50 are indicated. The figure shows only the portion of the gel that contains these proteins, other parts having been omitted for clarity.

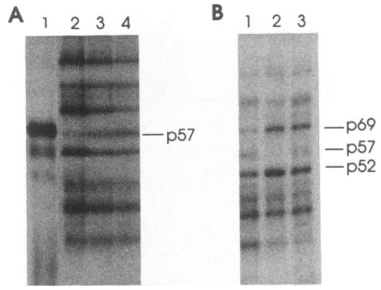

FIG. 6. Specific binding of p57 to the poliovirus 5′-NTR. (A) Increase in the specific binding of p57 to the poliovirus 5′-NTR by competition with rabbit rRNA. UV cross-linking of cellular proteins to [^{32}P]UTP-labeled RNA transcripts corresponding to nt 1 to 627 of the poliovirus 5′-NTR in the presence of 1 μg (lane 2), 2 μg (lane 3), or 3 μg (lane 4) of rabbit rRNA. Cellular proteins that bound to nt 260 to 488 of the EMCV 5′-NTR after UV cross-linking are shown in lane 1. Proteins were separated by electrophoresis on a 10 to 20% polyacrylamide gradient gel after completion of RNase digestion. P57 and p52 are indicated. (B) Reduction in the specific binding of p57 to the 5′-NTR of poliovirus by competition with a segment of the EMCV IRES. UV cross-linking of cellular proteins to [^{32}P]UTP-labeled RNA transcripts corresponding to nt 70 to 627 of the poliovirus 5′-NTR in the presence of 1.5 μg of rabbit rRNA (lane 1), 0.3 μg of unlabeled EMCV-specific RNA (corresponding to nt 260 to 488 of the 5′-NTR) (lane 2), or 0.3 μg of unlabeled poliovirus-specific RNA (corresponding to nt 559 to 624 of poliovirus type 2 [Lansing]) (lane 3). Proteins were separated by electrophoresis on a 10 to 20% polyacrylamide gradient gel after completion of RNase digestion. p57 and p52/p50 are indicated.

intensities of two bands, corresponding to a 69-kDa protein and to p50/p52 (which were not resolved in this gel), increased as a result of inclusion of 0.3 μg of unlabeled EMCV nt 260 to 488 RNA probe in the preincubation mixture (Fig. 6B, lane 2). The ratios of unlabeled EMCV and poliovirus competitor RNAs to [^{32}P]UTP-labeled poliovirus-specific probes in these experiments were 10:1 and 40:1, respectively.

The identity of the two p57 species was further confirmed by subcellular fractionation. The p57 species from RRL is predominantly a ribosome-associated protein (26). The p57 species that bound to the 5′-NTR of poliovirus was also found to be a ribosome-associated protein (that is, it occurred in the ribosomal salt wash fraction). Moreover, the two p57 species were both present in the ammonium sulfate 25 to 40% saturation fraction and copurified in all subsequent steps of the purification procedure that we have developed (unpublished data). The p57 species described here and that previously described as binding to the IRES of EMCV (8, 26) are therefore very probably the same moiety.

Specific inhibition of poliovirus translation by competition with the p57-binding domain of the EMCV IRES. Evidence presented above suggested that the p57 species that we have identified as binding to the poliovirus 5′-NTR are the same cellular proteins, described as p58 (8) or p57 (26), that interact with the 5′-NTR of EMCV. Since two point mutations in a stem-loop structure (stem-loop E) of the EMCV IRES abolished both p57 binding and translation of a downstream reporter gene, binding of p57 may be essential in EMCV translation (26). We therefore investigated the role of p57 in translation of poliovirus RNA.

Translation of P1 from transcripts of wt pPV1(M)5′-P1 (nt 70 to 3380) did not decrease as a result of addition of increasing amounts of rabbit rRNA (up to 2.5 μg) to 25-μl

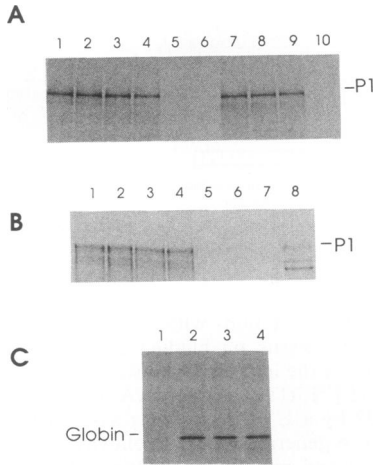

FIG. 7. Translation of poliovirus and globin mRNA in the presence of RNA competitors. (A) Specific reduction in the efficiency of translation of poliovirus RNA in HeLa cell lysate by competition with a segment of the EMCV IRES. Poliovirus-specific RNA was transcribed from wt pPV1(M)5′-P1, and 1.5 μg of mRNA was added to 25-μl aliquots of a HeLa cell lysate without competitor RNA (lane 1) or simultaneously with 1.5 μg (lane 2), 2 μg (lane 3), or 2.5 μg (lane 4) of rabbit rRNA, 0.4 μg (lane 5) or 0.8 μg (lane 6) of EMCV-specific RNA corresponding to nt 260 to 488 of the 5′-NTR, 0.4 μg (lane 7), 0.8 μg (lane 8), or 1.2 μg (lane 9) of RNA corresponding to nt 559 to 624 of poliovirus type 2 (Lansing). Lane 10 corresponds to lysate incubated without exogenous mRNA. Translation in the presence of [^{35}S]methionine was for 60 min at 30°C. Aliquots of translation assay mixture were separated by electrophoresis on a 10 to 20% polyacrylamide gradient gel. The P1 translation product is indicated. (B) Correlation between the ability of segments of the EMCV IRES to bind p57 and to act as competitive inhibitors of translation of poliovirus RNA in HeLa cell lysate. Poliovirus-specific RNA was transcribed from wt pPV1(M)5′-P1, and 1.5 μg of mRNA was added to 25-μl aliquots of a HeLa cell lysate without competitor RNA (lane 1) or simultaneously with 2 μg of rabbit rRNA (lane 2), 0.4 μg of EMCV-specific RNA (corresponding to nt 260 to 488 of the 5′-NTR and derived from pBS-ECAT403M1) (lane 3), 0.8 μg of the same EMCV-specific competitor RNA (lane 4), 0.8 μg of wt EMCV-specific RNA corresponding to nt 260 to 488 of the 5′-NTR (lane 5), or 0.8 μg of EMCV-specific RNA corresponding to nt 260 to 488 of the 5′-NTR and derived from pBS-ECAT403M2 (lane 6). Lane 7 corresponds to lysate incubated without exogenous mRNA. Lane 8 corresponds to a lysate of poliovirus-infected HeLa cells labeled in vivo with [^{35}S]methionine. Translation in the presence of [^{35}S]methionine was for 60 min at 30°C. Aliquots of translation assay mixture were separated by electrophoresis on a 10 to 20% polyacrylamide gradient gel. The P1 translation product is indicated. (C) Efficiency of translation of globin mRNA in HeLa cell lysate in the presence of a p57-binding segment of the EMCV IRES. Globin mRNA (0.5 μg) was added to 25-μl aliquots of a HeLa cell lysate without competitor RNA (lane 2) or simultaneously with 0.4 μg (lane 3) or 0.8 μg (lane 4) of EMCV-specific RNA corresponding to nt 260 to 488 of the 5′-NTR. Lane 1 corresponds to lysate incubated without exogenous mRNA. Translation in the presence of [^{35}S]methionine was for 60 min at 30°C. Aliquots of translation assay mixture were separated by electrophoresis on a 10 to 20% polyacrylamide gradient gel. The globin translation product is indicated.

aliquots of HeLa cell lysate to which 1.5 μg of poliovirus mRNA had been added simultaneously (Fig. 7A, compare lane 1 with lanes 2 through 4). Similarly, addition of up to 1.2 μg of the p52-binding domain of poliovirus type 2 (Lansing) (nt 559 to 624) did not affect the efficiency of translation of poliovirus RNA much, if at all (Fig. 7A, lanes 7 to 9).

However, addition of only 0.4 μg of EMCV-specific competitor RNA (corresponding to nt 260 to 488 of the 5′-NTR) to an aliquot of HeLa cell lysate simultaneously with poliovirus mRNA greatly reduced the efficiency of its translation (compare lanes 1 and 5 of Fig. 7A). Addition of 0.8 μg of this EMCV-specific RNA virtually abolished poliovirus translation (Fig. 7A, lane 6). In these experiments, the molar ratios of EMCV-specific competitor RNA to poliovirus mRNA were about 4:1 (lane 6) and 8:1 (lane 5).

We conducted further experiments to confirm that the reduction in the efficiency of poliovirus translation resulting from addition of this fragment of the EMCV IRES was due to its ability to bind p57. By means of point mutations, Jang and Wimmer (26) have constructed a version (pBS-ECAT403M1) of the EMCV IRES that is unable to bind p57 and a pseudorevertant (pBS-ECAT403M2) in which this ability has largely been restored (26). Addition of 0.4 or 0.8 μg of competitor RNA derived from pBS-ECAT403M1 (corresponding to nt 260 to 488 of the EMCV 5′-NTR) did not significantly affect the efficiency of translation of poliovirus RNA (Fig. 7B, lanes 3 and 4). Addition of 0.8 μg of equivalent competitor RNA derived from pBS-ECAT403M2 (the pseudorevertant) greatly reduced the efficiency of translation of poliovirus RNA (Fig. 7B, lane 6), although not to quite the extent observed following addition of an equal amount of equivalent wt RNA (lane 5). In these experiments, the molar ratios of EMCV-specific competitor RNA to poliovirus mRNA were about 4:1 (lane 3) and 8:1 (lanes 4, 5, and 6).

This segment (nt 260 to 488) of the EMCV IRES contains a stem-loop structure (55) that has been shown to bind p57 (26) with an affinity that enables it to outcompete the poliovirus 5′-NTR in binding p57 (Fig. 7A) under the conditions of the experiment described above. There is therefore a correlation between the ability of this EMCV RNA species to bind p57, to inhibit the binding of p57 to the poliovirus 5′-NTR, and to inhibit poliovirus translation. Conversely, neither rabbit rRNA nor the p52-binding domain of poliovirus competed with the poliovirus 5′-NTR in binding p57, and at the concentrations used, neither had a significant inhibitory effect on poliovirus translation. These results suggest that p57 plays an essential role in translation of poliovirus RNA.

The cellular p57 protein that binds to the 5′-NTRs of EMCV, FMDV, and poliovirus has properties that differ from those of all known mammalian translational initiation factors (8, 40; unpublished observations). The following additional evidence indicates that dependence on binding of p57 for efficient translation is not a ubiquitous phenomenon.

We translated globin mRNA (Boehringer Mannheim) in aliquots of the same batch of HeLa cell lysate used in the experiment described immediately above, in the absence of exogenous competitor RNA (Fig. 7C, lane 2) and in the presence of either 0.4 μg (lane 3) or 0.8 μg (lane 4) of the EMCV-specific competitor RNA described above. It is apparent that addition of a concentration of EMCV-specific competitor RNA that virtually abolished poliovirus translation had little effect on translation of globin mRNA.

DISCUSSION

The initiation of translation of picornavirus RNA is cap independent and involves internal entry of ribosomes, but details of the mechanism by which it occurs are still largely unknown. We have therefore begun to characterize the *cis*-acting RNA elements and *trans*-acting protein factors involved in this process. As a result of the experiments described above, we have found that nt 559 to 565 constitute an important *cis*-acting RNA element for translation of poliovirus in HeLa cell lysate. We have also found that a cellular protein, p57, appears to be an important *trans*-acting factor in the translation of poliovirus in vitro. A similar observation has been made previously regarding translation controlled by the EMCV IRES (26).

The *cis*-acting RNA element is part of a larger pyrimidine-rich tract that occurs in all picornaviruses (25) (Table 1). In poliovirus, the residues within this *cis*-acting element that are the most critical for translation in vitro correspond precisely to those identified as being essential for viral infectivity (19). Our results support the suggestion that the oligopyrimidine tract plays an important role in translation of poliovirus mRNA. Several distinct functions can be envisaged for this tract that are compatible with initiation of translation by internal entry of ribosomes. The oligopyrimidine tract in picornaviruses was first noted by Beck et al. (6), who suggested that in FMDV this element shows a significant complementarity to a purine-rich sequence close to the 3′ end of eukaryotic 18S rRNA. Such an interaction could promote binding of the 40S ribosomal subunit, in a manner analogous to the Shine-Dalgarno sequence of prokaryotic mRNAs. Similar complementarity occurs between the 5′ half of the oligopyrimidine tract of poliovirus (nt 559 to 567; 5′-UUUCCUUUU-3′) and nt 1857 to 1863 (3′-AGGAAGG-5′) of mammalian 18S rRNA (41). A potential interaction of this type is appealing because the poliovirus residues that are most sensitive to substitution would normally interact most strongly with 18S rRNA. However, since mutation of the oligopyrimidine tract did not affect the efficiency of poliovirus translation in Krebs-2 cell lysate or RRL despite the purine-rich regions at the 3′ end of murine, rabbit, and human 18S rRNA species being identical in sequence (41), this explanation may in itself not be sufficient. It should be noted that the oligopyrimidine tract at nt 558 to 564 occurs nearly 200 nt upstream of the initiating AUG codon in poliovirus RNA, for which reason it can hardly function in a manner identical to that of the Shine-Dalgarno sequence. However, the oligopyrimidine tract does occur just 18 nt upstream of an essential, albeit noncoding, AUG triplet (49). It is possible that this Y_n-X_m-AUG sequence (in which n pyrimidine [Y] residues are separated from the AUG by a number [m] of any nucleotide [X]) may be an essential signal for internal ribosomal entry (25).

Substitutions that effectively resulted in a movement of the *cis*-acting element to a position 4 nt further away from the 5′ terminus (mutant 10) did not compensate for the reduction in the efficiency of translation caused by inactivation of the original *cis*-acting element. We speculate that the functional importance of this element might be related to its position relative to those of other *cis*-acting elements within the IRES. These could include protein-binding sites, the initiation codon and its immediate context, or the aforementioned AUG triplet (nt 584 to 586 in poliovirus type 1 [Mahoney]), whose position relative to the pyrimidine-rich tract is relatively constant in all picornaviruses (25). The importance of this triplet for translation of poliovirus has been demonstrated (49). The oligopyrimidine tract may be involved in previously uncharacterized secondary or tertiary interactions (for example, involving residues downstream of nt 630, which have not been considered in existing models) that are disrupted by mutation. Structural perturbation would not be expected to result in a tissue-specific difference in the efficiency of translation unless it resulted in reduced

affinity of the 5′-NTR for a specific protein factor. We investigated this possibility by using a UV cross-linking assay and [^{32}P]UTP-labeled poliovirus-specific RNA transcripts. As a result, we were able to confirm binding of p52 to a segment of the poliovirus 5′-NTR encompassing nt 559 to 624, but neither its binding nor that of any other protein appeared to be altered as a result of the substitutions made within the pyrimidine-rich tract. Although UV cross-linking reactions were done under conditions that had been optimized for initiation of translation of poliovirus, we do not rule out the possibility that some bona fide protein-RNA interactions were not detected because of the intrinsic limitations of the assay.

Using UV cross-linking, we did detect the specific interaction of a protein species of ca. 57 kDa (p57) with the poliovirus 5′-NTR. It does not correspond to any protein previously reported as binding to the poliovirus 5′-NTR (5, 11, 42, 44). p57 is the same protein species recently described as binding to the EMCV 5′-NTR (8, 26), and its physical properties lead us to conclude that it is also the protein that binds to the 5′-NTR of FMDV (40). The data described here and by Jang and Wimmer (26) indicate that this is a novel *trans*-acting cellular factor that plays a critical role in the cap-independent mechanism of picornavirus translation. p57 occurs largely in the ribosomal salt wash, indicating that it is a ribosome-bound protein, and as such it may mediate binding of ribosomes to the IRES by virtue of its affinity for a specific RNA sequence or structural element. We have shown that p57 could be UV cross-linked to nt 1 to 627 but not nt 1 to 220 of the poliovirus 5′-NTR; experiments to define the binding site more precisely are in progress. It is highly likely that the involvement of p57 in translation is not unique to picornaviral mRNAs. Although translation of globin mRNA apparently does not require p57, there may nevertheless be a class of cellular mRNAs whose translation is dependent on this protein. We do not anticipate that translation of such mRNAs necessarily occurs as a result of cap-independent internal entry of ribosomes.

There are clear differences in the characteristics of translation of EMCV and poliovirus mRNAs, especially with regard to their relative Mg^{2+} and K^{+} optima and their abilities to be translated accurately and efficiently in some cell lysates (e.g., RRL [21, 24]). Nevertheless, the results presented above and in a recent report from this laboratory (26) indicate that the mechanisms adopted by the two viruses to promote internal entry of ribosomes have many common features. Both RNAs contain a pyrimidine-rich tract located close to or at the 3′ border of the IRES, the 5′ half of which functions as an important *cis*-acting element. Second, both RNAs can be cross-linked to p57, which appears to be an essential *trans*-acting protein factor. Although we have not yet determined the site(s) or determinants of binding of p57 to the 5′-NTR of poliovirus, it appears that the EMCV IRES can outcompete the poliovirus IRES in binding p57. This may contribute to the exceptionally efficient translation of EMCV RNA in circumstances in which p57 may be a limiting factor. For example, p57 is present in very low concentration in RRL (40; unpublished observations), which is a cell lysate in which poliovirus is translated relatively less efficiently than EMCV. Some differences between translation of EMCV and poliovirus may therefore reflect different affinities for critical and possibly limiting factors, possibly resulting from adaptation of these viruses to specific hosts or tissues. It is tempting to speculate that the reduced efficiency of translation of attenuated strains of poliovirus may be due at least in part to reduced affinity for p57. At least two additional factors, termed initiation correction factor and p52, have recently been implicated in promoting the efficient and accurate translation of poliovirus (42, 73). The physical and biochemical properties of p57 indicate that it is distinct from both.

ACKNOWLEDGMENTS

We thank J. Dunn for the T7 RNA polymerase; S. K. Jang for the gift of pBS-ECAT, pBS-ECAT403M1, and pBS-ECAT403M2; J. Harber for invaluable assistance in site-directed mutagenesis; A. Palmenberg for communicating unpublished nucleotide sequences and sequence alignments; P. Kissel for synthesis of oligonucleotides; and C. Helmke for photography.

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.

REFERENCES

1. **Agol, V. I.** The 5′-untranslated region of picornaviral genomes. Adv. Virus. Res., in press.
2. **Agol, V. I., S. G. Drozdov, T. A. Ivannikova, M. S. Kolesnikova, M. B. Korolev, and E. A. Tolskaya.** 1989. Restricted growth of attenuated poliovirus strains in cultured cells of a human neuroblastoma. J. Virol. **63**:4034–4038.
3. **Almond, J. W.** 1987. The attenuation of poliovirus neurovirulence. Annu. Rev. Microbiol. **41**:153–180.
4. **AlSaadi, S., S. Hassard, and G. Stanway.** 1989. Sequences in the 5′ noncoding region of human rhinovirus type 14 RNA that affect *in vitro* translation. J. Gen. Virol. **70**:2799–2804.
5. **Andino, R., G. E. Rieckhof, and D. Baltimore.** 1990. A functional ribonucleoprotein complex forms around the 5′ end of poliovirus RNA. Cell **63**:369–380.
6. **Beck, E., S. Forss, K. Strebel, R. Cattaneo, and G. Feil.** 1983. Structure of the FMDV translation initiation site and of the structural protein. Nucleic Acids Res. **11**:7873–7885.
7. **Bienkowska-Szewcysk, K., and E. Ehrenfeld.** 1988. An internal 5′-noncoding region required for translation of poliovirus RNA in vitro. J. Virol. **62**:3068–3072.
8. **Borovjagin, A. V., A. G. Evstafieva, T. Y. Ugarova, and I. N. Shatsky.** 1990. A factor that specifically binds to the 5′-untranslated region of encephalomyocarditis virus RNA. FEBS Lett. **261**:237–240.
9. **Brown, B. A., and E. Ehrenfeld.** 1979. Translation of poliovirus RNA *in vitro*: changes in cleavage pattern and initiation sites by ribosomal salt wash. Virology **97**:396–405.
10. **Chapman, N. M., S. Tracy, C. J. Gauntt, and U. Fortmueller.** 1990. Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. J. Clin. Microbiol. **28**:843–850.
11. **Del Angel, R. M., A. G. Papavassilliou, C. Fernandez-Tomas, S. J. Silverstein, and V. R. Racaniello.** 1989. Cell proteins that bind to multiple sites within the 5′-untranslated region of poliovirus RNA. Proc. Natl. Acad. Sci. USA **86**:8299–8303.
12. **de Moor, C. H., H. A. A. van Heugten, and H. O. Voorma.** 1990. Characterization of messenger ribonucleoprotein particles. Mol. Biol. Rep. **14**:57–60.
13. **Dorner, A. J., L. F. Dorner, G. R. Larsen, E. Wimmer, and C. W. Anderson.** 1982. Indentification of the initiation site of poliovirus polyprotein synthesis. J. Virol. **42**:1017–1028.
14. **Dorner, A. J., B. L. Semler, R. J. Jackson, R. Hanecak, E. Duprey, and E. Wimmer.** 1984. In vitro translation of poliovirus RNA: utilization of internal initiation sites in reticulocyte lysate. J. Virol. **50**:507–514.
15. **Dreyfuss, G.** 1986. Structure and function of nuclear and cytoplasmic ribonucleoprotein particles. Annu. Rev. Cell Biol. **2**:459–498.
16. **Dunn, J. J., and F. W. Studier.** 1983. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. J. Mol. Biol. **166**:477–535. (Erratum, **175**: 1539–1546, 1984.)
17. **Hellen, C. U. T., H.-G. Kräusslich, and E. Wimmer.** 1989. Proteolytic processing of polyproteins in the replication of RNA

viruses. Biochemistry **28**:9881–9890.
18. **Howell, M. T., A. Kaminski, and R. J. Jackson.** 1990. Unique features of initiation of picornavirus RNA translation, p. 144–151. *In* M. Brinton and F. Z. Heinz (ed.), New aspects of positive-strand viruses. American Society for Microbiology, Washington, D.C.
19. **Iizuka, N., M. Kohara, K. Hagino-Yamagishi, S. Abe, T. Komatsu, K. Tago, M. Arita, and A. Nomoto.** 1989. Construction of less neurovirulent polioviruses by introducing deletions into the 5′-noncoding sequence of the genome. J. Virol. **63**:5354–5365.
20. **Innis, M. A., and D. H. Gelfland.** 1990. Optimization of PCRs, p. 3–12. *In* M. A. Innis, D. H. Gelfland, J. J. Sninsky, and T. J. White (ed.), PCR protocols. Academic Press, Inc., New York.
21. **Jackson, R. J.** 1989. Comparison of encephalomyocarditis virus and poliovirus with respect to translation initiation and processing in vitro, p. 51–71. *In* B. L. Semler and E. Ehrenfeld (ed.), Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington, D.C.
22. **Jackson, R. J., M. T. Howell, and A. Kaminski.** 1990. The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem. Sci. **15**:477–483.
23. **Jang, S. K., M. V. Davies, R. J. Kaufman, and E. Wimmer.** 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5′ nontranslated region of encephalomyocarditis virus RNA in vivo. J. Virol. **63**:1651–1660.
24. **Jang, S. K., H.-G. Kräusslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62**:2636–2643.
25. **Jang, S. K., T. V. Pestova, C. U. T. Hellen, G. W. Witherell, and E. Wimmer.** 1991. Cap-independent translation of picornaviral mRNAs: structure and function of the internal ribosomal entry site. *In* R. E. Thach (ed.), Translationally regulated genes in higher eukaryotes, in press. S. Karger, Basel.
26. **Jang, S. K., and E. Wimmer.** 1990. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57 kD RNA-binding protein. Genes Dev. **4**:1560–1572.
27. **Koch, F., and G. Koch.** 1985. The molecular biology of poliovirus. Springer-Verlag, Vienna.
28. **Kozak, M.** 1986. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proc. Natl. Acad. Sci. USA **83**:2850–2854.
29. **Kozak, M.** 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15**:8125–8133.
30. **Kräusslich, H.-G., C. Hölscher, Q. Reuer, J. Harber, and E. Wimmer.** 1990. Myristoylation of the poliovirus polyprotein is required for proteolytic processing of the capsid and for viral infectivity. J. Virol. **64**:2433–2436.
31. **Kuge, S., and A. Nomoto.** 1987. Construction of viable deletion and insertion mutants of the Sabin strain of type 1 poliovirus: function of the 5′ noncoding sequence in viral replication. J. Virol. **61**:1478–1487.
32. **Kühn, R., N. Luz, and E. Beck.** 1990. Functional analysis of the internal translation initiation site of foot-and-mouth disease virus. J. Virol. **64**:4625–4631.
33. **Kunkel, T.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
34. **La Monica, N., and V. R. Racaniello.** 1989. Differences in replication of attenuated and neurovirulent polioviruses in human neuroblastoma cell line SH-SY5Y. J. Virol. **63**:2357–2360.
35. **Larsen, G. R., B. L. Semler, and E. Wimmer.** 1981. Stable hairpin structure within the 5′-terminal 85 nucleotides of poliovirus RNA. J. Virol. **37**:328–335.
36. **Le, S.-Y., and M. Zuker.** 1990. Common structures of the 5′-noncoding RNA in enteroviruses and rhinoviruses. Thermodynamical stability and statistical significance. J. Mol. Biol. **216**:729–741.
37. **Lee, C.-K., and E. Wimmer.** 1988. Proteolytic processing of poliovirus polyprotein: elimination of $2A^{pro}$-mediated, alternative cleavage of polypeptide 3CD by *in vitro* mutagenesis. Virology **166**:405–414.
38. **Lee, K. A. W., and N. Sonenberg.** 1982. Inactivation of cap-binding proteins accompanies the shut-off of host protein synthesis by poliovirus. Proc. Natl. Acad. Sci. USA **79**:3447–3451.
39. **Lopez-Guerrero, J.-A., L. Carrasco, F. Martinez-Abarca, M. Fresno, and M. A. Alonso.** 1989. Restriction of poliovirus RNA translation in a human monocytic cell line. Eur. J. Biochem. **186**:577–582.
40. **Luz, N., and E. Beck.** 1990. A cellular 57kDa protein binds to two regions of the internal translation initiation site of foot-and-mouth disease virus. FEBS Lett. **269**:311–314.
41. **McCallum, F. S., and B. E. H. Maden.** 1985. Human 18S ribosomal RNA sequence inferred from DNA sequence. Variations in 18S sequences and secondary modification patterns between vertebrates. Biochem. J. **232**:725–733.
42. **Meerovitch, K., J. Pelletier, and N. Sonenberg.** 1989. A cellular protein that binds to the 5′-noncoding region of poliovirus RNA: implications for internal translation initiation. Genes Dev. **3**:1026–1034.
43. **Milligan, J. F., D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck.** 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res. **15**:8783–8798.
44. **Najita, L., and P. Sarnow.** 1990. Oxidation-reduction-sensitive interaction of a cellular 50-kDa protein with an RNA hairpin in the 5′-noncoding region of the poliovirus genome. Proc. Natl. Acad. Sci. USA **87**:5846–5850.
45. **Nicklin, M. J. H., H.-G. Kräusslich, H. Toyoda, J. J. Dunn, and E. Wimmer.** 1987. Poliovirus polypeptide precursors. Expression in vitro and processing by 3C and 2A proteinases. Proc. Natl. Acad. Sci. USA **84**:4002–4006.
46. **Nomoto, A., and E. Wimmer.** 1987. Genetic studies of the antigenicity and the attenuated phenotype of poliovirus, p. 107–134. *In* Molecular basis of virus diseases (Society for General Microbiology Symposium 40 Series). Cambridge University Press, Cambridge.
47. **Palmenberg, A. C., E. M. Kirby, M. R. Janda, N. L. Drake, G. M. Drake, G. M. Duke, K. F. Potratz, and M. S. Collett.** 1984. The nucleotide and deduced amino acid sequences of the encephalomyocarditis viral polyprotein coding region. Nucleic Acids Res. **12**:2969–2985.
48. **Paul, A. V., T. Hiroomi, K. von der Helm, T. Wissel, R. Kiehn, E. Wimmer, and F. Deinhardt.** 1987. The entire nucleotide sequence of the genome of human hepatitis A virus (isolate MBB). Virus Res. **8**:153–171.
49. **Pelletier, J., M. E. Flynn, G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Mutational analysis of upstream AUG codons of poliovirus RNA. J. Virol. **62**:4483–4492.
50. **Pelletier, J., G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Cap-independent translation of poliovirus mRNA is conferred by sequence elements within the 5′ noncoding region. Mol. Cell. Biol. **8**:1103–1112.
51. **Pelletier, J., G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Translational efficiency of poliovirus mRNA: mapping inhibitory *cis*-acting elements within the 5′-noncoding region. J. Virol. **62**:2219–2227.
52. **Pelletier, J., and N. Sonenberg.** 1985. Insertion mutagenesis to increase secondary structure within the 5′ noncoding region of eukaryotic mRNA reduces translational efficiency. Cell **40**:515–526.
53. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334**:320–325.
54. **Phillips, B. A., and A. Emmert.** 1986. Modulation of the expression of poliovirus proteins in reticulocyte lysates. Virology **148**:255–267.
55. **Pilipenko, E. V., V. M. Blinow, B. K. Chernov, T. M. Dimitrieva, and V. I. Agol.** 1989. Conservation of the secondary structure elements of the 5′-untranslated region of cardio- and aphthovirus RNAs. Nucleic Acids Res. **17**:5701–5711.
56. **Pilipenko, E. V., V. M. Blinow, L. I. Romanova, A. N. Sinyakov, S. V. Maslova, and V. I. Agol.** 1989. Conserved structural

domains in the 5′-untranslated region of picornaviral genomes: an analysis of the segment controlling translation and neurovirulence. Virology **168**:201–209.
57. **Rhoads, R. E.** 1988. Cap recognition and the entry of mRNA into the protein synthesis initiation cycle. Trends Biochem. Sci. **13**:52–56.
58. **Rose, J. K., H. Trachsel, K. Leong, and D. Baltimore.** 1978. Inhibition of translation by poliovirus: inactivation of a specific initiation factor. Proc. Natl. Acad. Sci. USA **75**:2732–2736.
59. **Ruiz-Linares, A., M. Bouloy, M. Girard, and A. Cahour.** 1989. Modulation of the *in vitro* translational efficiencies of yellow fever virus mRNAs: interactions between coding and noncoding regions. Nucleic Acids Res. **17**:2463–2476.
60. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
61. **Sangar, D. V., S. E. Newton, D. J. Rowlands, and B. E. Clarke.** 1987. All foot and mouth disease virus serotypes initiate protein synthesis at two separate AUGs. Nucleic Acids Res. **15**:3305–3315.
62. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
63. **Schreier, M. H., and T. Staehelin.** 1973. Initiation of mammalian protein synthesis: the importance of ribosome and initiation factor quality for the efficiency of *in vitro* systems. J. Mol. Biol. **73**:329–349.
64. **Seechurn, P., N. J. Knowles, and J. W. McCauley.** 1990. The complete nucleotide sequence of a pathogenic swine vesicular disease virus. Virus Res. **16**:255–274.
65. **Shih, D. S., C. T. Shih, O. Kew, M. Pallansch, R. R. Rueckert, and P. Kaesberg.** 1978. Cell-free synthesis and processing of the proteins of poliovirus. Proc. Natl. Acad. Sci. USA **75**:5807–5811.
66. **Skinner, M. A., V. R. Racaniello, G. Dunn, J. Cooper, P. D. Minor, and J. W. Almond.** 1989. New model for the secondary structure of the 5′ noncoding RNA of poliovirus is supported by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J. Mol. Biol. **207**:379–392.
67. **Sonenberg, N.** 1987. Regulation of translation by poliovirus. Adv. Virus Res. **33**:175–204.
68. **Sonenberg, N.** 1990. Poliovirus translation. Curr. Top. Microbiol. Immunol. **161**:23–47.
69. **Stanway, G.** 1990. Structure, function and evolution of picornaviruses. J. Gen. Virol. **71**:2483–2501.
70. **Svitkin, Y. V., N. Cammack, P. D. Minor, and J. W. Almond.** 1990. Translation deficiency of the Sabin type 3 poliovirus genome: association with an attenuating mutation $C_{472} \rightarrow U$. Virology **175**:103–109.
71. **Svitkin, Y. V., V. N. Lyapustin, V. A. Lashkevich, and V. I. Agol.** 1984. Differences between translation products of tick-borne encaphalitis virus RNA in cell-free systems from Krebs-2 cells and rabbit reticulocytes: involvement of membranes in the processing of nascent precursors of flavivirus structural proteins. Virology **135**:536–541.
72. **Svitkin, Y. V., S. V. Maslova, and V. I. Agol.** 1985. The genomes of attenuated and virulent poliovirus strains differ in their *in vitro* translation efficiencies. Virology **147**:243–252.
73. **Svitkin, Y. V., T. V. Pestova, S. V. Maslova, and V. I. Agol.** 1988. Point mutations modify the response of poliovirus RNA to a translation initiation factor. A comparison of neurovirulent and attenuated strains. Virology **166**:394–404.
74. **Toyoda, H., M. Kohara, Y. Kataoka, T. Sugunuma, T. Omata, N. Imura, and A. Nomoto.** 1984. The complete nucleotide sequence of all three poliovirus serotype genomes: implication for the genetic relationship, gene function and antigenic determinants. J. Mol. Biol. **174**:561–585.
75. **Van der Werf, S., J. Bradley, E. Wimmer, F. W. Studier, and J. J. Dunn.** 1986. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:2330–2334.
76. **Wimmer, E.** 1982. Genome-linked proteins of viruses. Cell **28**:199–201.