

## Internal Binding of Eucaryotic Ribosomes on Poliovirus RNA: Translation in HeLa Cell Extracts

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**Translation initiation on poliovirus mRNA in poliovirus-infected cells has been shown to occur by internal binding of ribosomes to the 5' noncoding region (J. Pelletier and N. Sonenberg, *Nature [London]* 334:320-325, 1988). Here we show that internal ribosome binding can occur in HeLa cell extracts in vitro. Internal binding to the 5' noncoding region of poliovirus mRNA in a bicistronic context was independent of the upstream open reading frame and did not require poliovirus proteins.**

Translation initiation of picornavirus mRNAs appears to be different from that of most eucaryotic cellular and viral mRNAs. Picornavirus mRNAs are not blocked at their 5' ends by a cap structure (m<sup>7</sup>GpppN, where N is any nucleotide), and their translation proceeds by a cap-independent mechanism (for reviews, see references 3 and 14). Translation initiation in eucaryotes is believed to occur by ribosome binding at or near the 5' cap structure (which facilitates ribosome binding), followed by ribosome scanning of the 5' noncoding region until an appropriate AUG initiator is encountered (6). Poliovirus, encephalomyocarditis virus, and most probably all other picornavirus mRNAs are translated by a different mechanism, whereby ribosomes do not scan from the 5' end of the mRNA but bind directly to an internal sequence (at least 320 nucleotides downstream from the 5' end in the case of poliovirus) in the 5' noncoding region and are then translocated to the initiator AUG to initiate translation (5, 11).

Experiments demonstrating internal binding of ribosomes to the 5' noncoding region of poliovirus mRNA were previously carried out in vivo and in vitro in poliovirus-infected cells (11). In the previous study (11), the poliovirus 5' untranslated region (UTR) served as the intercistronic spacer in a bicistronic mRNA and allowed translation of the second cistron under conditions in which translation of the first cistron was curtailed. In the present study we extend these results by showing that internal initiation can take place in the absence of poliovirus proteins in uninfected HeLa cell extracts.

To address the mechanism of translation of poliovirus RNA in uninfected cells and to extend our previous experiments (10, 11), we constructed a second generation of bicistronic mRNAs consisting of  $\sigma$ S (coding sequence for the  $\sigma$ S protein derived from the S1 gene of reovirus [13]) as the first open reading frame (ORF) and the chloramphenicol acetyltransferase (CAT) gene (*cat*) as the second ORF. In a second construct, we inserted the 5' noncoding region of poliovirus as the intercistronic region between these genes. These constructs are different from the ones we have used previously in which the first ORF was the thymidine kinase gene of herpes simplex virus type 1 (11).

The plasmids constructed for this study are shown in Fig.

1. Plasmids CAT, P2CAT, TK/CAT, and TK/P2CAT have been described previously (10, 11). Plasmid  $\sigma$ S, originally named pSP64/S1 (13), was created by inserting a *Bam*HI-*Bgl*II portion of the reovirus (type 2, Dearing strain) S1 gene into the *Bam*HI site of pSP64 (13). In this construct, the 5'-proximal AUG of the reovirus S1 mRNA, which directs the synthesis of  $\sigma$ 1 (~46 kilodaltons [kDa]), is deleted and the new 5'-proximal AUG encodes a ~16-kDa polypeptide, which is  $\sigma$ S (13). Plasmid  $\sigma$ S was cleaved with *Eco*RV (which cleaves 64 nucleotides downstream of the  $\sigma$ S termination codon) and treated with calf intestinal alkaline phosphatase. The *cat* and P2CAT genes were excised from plasmids CAT and P2CAT, respectively, by restriction with *Hind*III and *Hpa*I, and purified on a low-melting-point agarose gel (8). The *Hind*III site was rendered flush by treatment with DNA polymerase I (Klenow fragment) and blunt-end ligated into plasmid  $\sigma$ S with T4 DNA ligase.

For in vitro transcriptions, plasmids were linearized with *Sma*I. Transcriptions, mRNA quantitations, and analysis were carried out as previously described (10). Translation in HeLa extracts was carried out according to Rose et al. (12). Translation extracts from HeLa cells were prepared essentially as described before (12), with minor modifications (9). Infection of cells with poliovirus type 1 (Mahoney strain) was done as described before (9). Translation products were analyzed on 10 to 15% polyacrylamide-sodium dodecyl sulfate (SDS) gels (7). Gels were fixed in 40% methanol-7.5% acetic acid, treated with *En*<sup>3</sup>Hance, and exposed against an X-ray film at -70°C.

Figure 2 shows the translation products of  $\sigma$ S/CAT and  $\sigma$ S/P2CAT mRNAs in uninfected HeLa cell extracts, in the presence and absence of the cap analog m<sup>7</sup>GDP. This compound inhibits translation initiation that is mediated by the cap structure but should not inhibit translation that initiates by internal binding of ribosomes to the poliovirus 5' noncoding region. Lanes 2 and 3 show the translation products obtained from *cat* (~28 kDa) and  $\sigma$ S (~14 kDa) mRNAs, respectively. Translation of  $\sigma$ S/CAT mRNA showed that expression of the 5'-proximal cistron ( $\sigma$ S) was cap dependent, since its translation was enhanced 10-fold by the presence of a methylated cap structure (compare lanes 5 and 4) and translation was inhibited (10-fold) by the addition of m<sup>7</sup>GDP (compare lanes 6 and 5). Likewise, translation of the second cistron (CAT) was stimulated (sixfold) by the presence of a methylated cap structure (compare lanes 5 and

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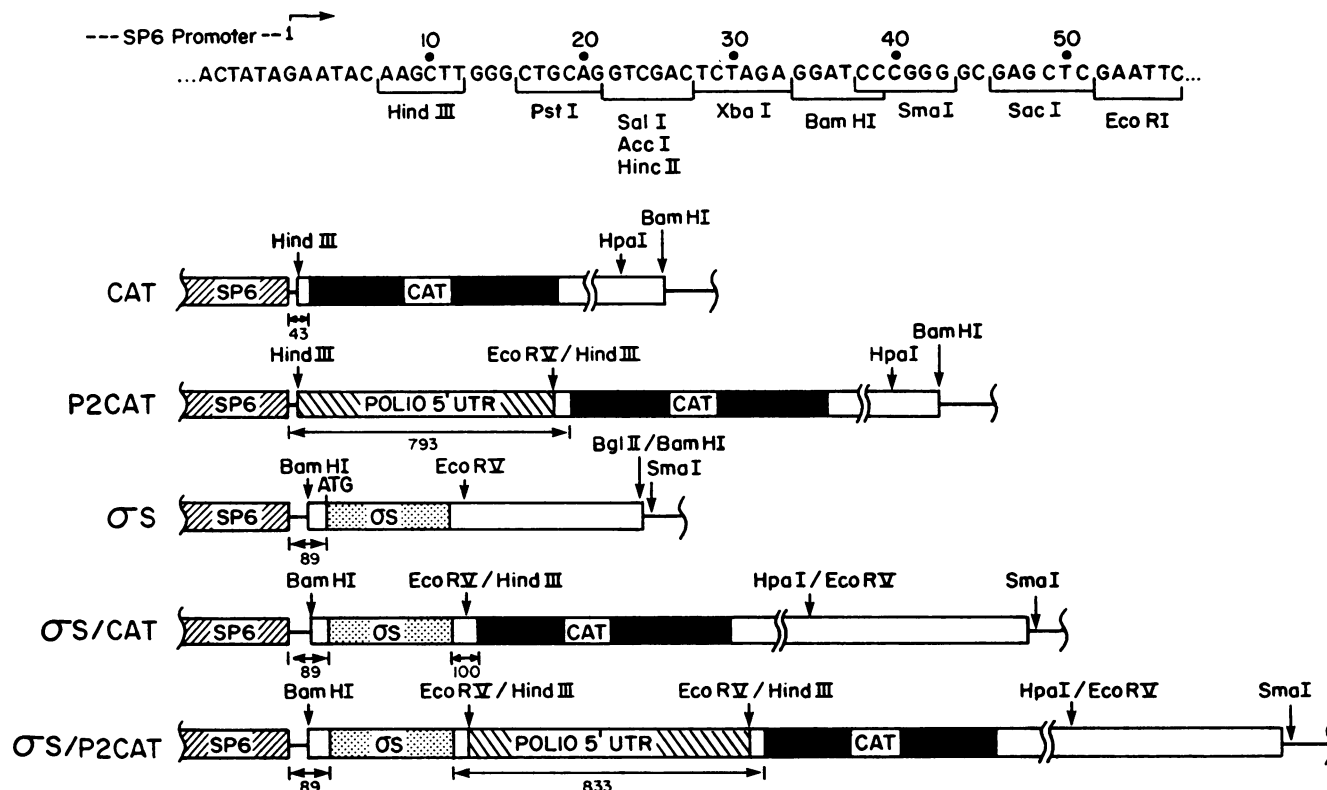


FIG. 1. Structure of bicistronic genes constructed by fusing the  $\sigma$ S and *cat* ORFs. The polylinker region of pSP64 is shown at the top of the diagram as a reference to the restriction sites used to create the bicistronic constructs. The right-angled arrow denotes the start and direction of transcription of SP6 polymerase. The numbers below the constructs represent distances in nucleotides. The blank box denotes 5' and 3' UTRs, and the thin line represents sequences from pSP64. The stippled box represents the  $\sigma$ S coding region; the hatched box is the poliovirus 5' UTR; and the blackened box is the *cat* ORF.

4) and inhibited (approximately fivefold) by the cap analog  $m^7$ GDP (compare lanes 6 and 5). These results demonstrate that synthesis of CAT protein from  $\sigma$ S/CAT mRNA is due to reinitiating ribosomes, whose initial binding to the mRNA is cap mediated.

When the poliovirus 5' noncoding region was inserted between  $\sigma$ S and the *cat* cistrons (Fig. 1) the translation of CAT from GpppG-terminated mRNA was significantly higher (eightfold) than that from  $\sigma$ S/CAT mRNA (compare lanes 7 and 4). This is in sharp contrast to the absence of stimulation of translation of  $\sigma$ S protein (twofold lower). Translation of capped methylated  $\sigma$ S/P2CAT mRNA showed enhancement of  $\sigma$ S synthesis compared with that from GpppG-terminated  $\sigma$ S/P2CAT mRNA (4.5-fold), whereas the level of CAT synthesized was not changed (compare lanes 8 and 7). Addition of  $m^7$ GDP had no effect on translation of the *cat* ORF from the capped methylated  $\sigma$ S/P2CAT mRNA, but inhibited (threefold) the translation of  $\sigma$ S from the 5' ORF of  $\sigma$ S/P2CAT (compare lanes 9 and 8). The data are summarized in Table 1. Thus, translation of the *cat* ORF under the control of the poliovirus 5' noncoding region is clearly independent of that of the upstream  $\sigma$ S ORF in an uninfected HeLa cell extract.

We have also performed translation experiments in HeLa extracts prepared from poliovirus-infected cells to ascertain the cap independence of translation of the *cat* ORF from  $\sigma$ S/P2CAT mRNA as was shown for TK/P2CAT (11). The cap-dependent CAT,  $\sigma$ S, and thymidine kinase (TK)

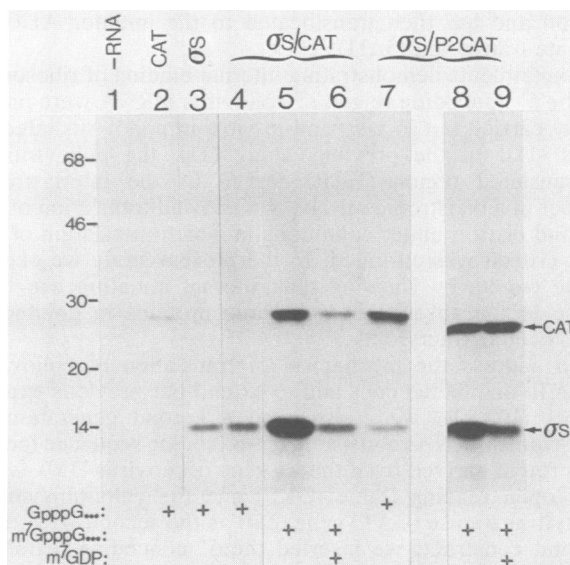


FIG. 2. Translation of bicistronic mRNAs in an uninfected HeLa cell extract. Translations were performed at an mRNA concentration of 30  $\mu$ g/ml at 30°C for 60 min. Samples were processed for electrophoresis and visualized by fluorography as described before (10). Lanes containing unmethylated (GpppG) and methylated ( $m^7$ GpppG) capped mRNAs are indicated.  $m^7$ GDP (50  $\mu$ M final concentration) was added where indicated. The positions of migration of the  $\sigma$ S and CAT proteins are shown. Molecular mass markers are shown in kilodaltons.

TABLE 1. Synthesis of  $\sigma$ S and CAT from bicistronic mRNAs in an uninfected HeLa cell extract

mRNA	m <sup>7</sup> GDP	Ratio, CAT/ $\sigma$ S
GpppG... $\sigma$ S/CAT	—	0.2
m <sup>7</sup> GpppG... $\sigma$ S/CAT	—	0.1
m <sup>7</sup> GpppG... $\sigma$ S/CAT	+	0.3
GpppG... $\sigma$ S/P2CAT	—	2.5
m <sup>7</sup> GpppG... $\sigma$ S/P2CAT	—	0.7
m <sup>7</sup> GpppG... $\sigma$ S/P2CAT	+	1.7

<sup>a</sup> The autoradiograph in Fig. 2 was scanned by soft laser densitometry (LKB). Values obtained were normalized for the different methionine contents of  $\sigma$ S and CAT, and the ratio of CAT to  $\sigma$ S protein is shown.

mRNAs were not translated in these extracts (Fig. 3, lanes 2, 4, and 7) because of the inactivation of the cap-binding complex, eIF-4F (3, 4, 14). Fusion of the poliovirus 5' UTR to *cat* permitted efficient translation (lane 3), as reported previously (10), demonstrating that this sequence was functional in the extracts from poliovirus-infected cells. The *cat* ORF was not translated when it was juxtaposed immediately downstream of the  $\sigma$ S ORF (lane 5) or TK ORF (lane 8), emphasizing the need for 5' cap-mediated initiation to express  $\sigma$ S or CAT from  $\sigma$ S/CAT mRNA. However, in the construct in which the poliovirus 5' UTR was inserted as the intercistronic region between  $\sigma$ S and CAT, synthesis of  $\sigma$ S was still undetectable, but translation of CAT was almost as efficient (1.5- to 2-fold less) as the translation of TK/P2CAT and P2CAT mRNAs (compare lanes 6 and 9 or 3). This result supports the contention that translation of CAT occurred by internal initiation which was separate from and independent of initiation at the 5' end and that the nature of the upstream ORF had no effect on CAT translation.

The results presented here extend and support the conclusions we have drawn previously (11), that the poliovirus 5' UTR serves as a ribosome landing pad (RLP) for translation initiation. Binding of ribosomes to the RLP is completely independent of two different upstream sequences, which in this study consisted of  $\sigma$ S and in the previous study of the

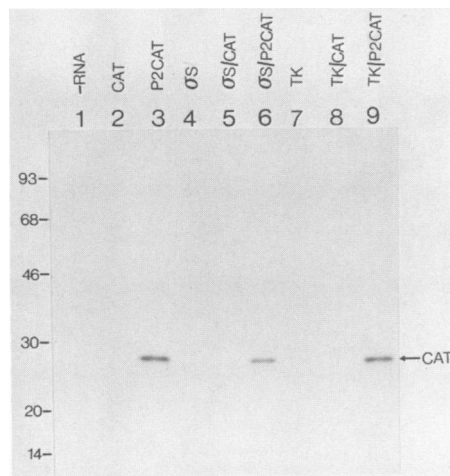


FIG. 3. Translation of bicistronic mRNAs in a poliovirus-infected HeLa cell extract. Translations were performed at an mRNA concentration of 30  $\mu$ g/ml at 30°C for 60 min. Samples were processed for electrophoresis and visualized by fluorography as described before (10). Molecular mass markers are shown in kilodaltons.

TK of herpes simplex virus type 1 (11). In addition, since the RLP functions in uninfected HeLa cell extracts, this domain does not require *trans*-acting poliovirus proteins. Thus, the machinery required for the recognition of the poliovirus RLP is present in uninfected cells. It is unlikely that this machinery exists solely for the purpose of supporting poliovirus or other picornavirus replication. It is possible that internal translation initiation occurs on cellular mRNAs. The extent to which this occurs might vary among mRNAs, depending on different structural elements within the 5' UTRs. In this respect, it is of interest that poliovirus infection of HeLa cells in the presence of guanidine results in the complete proteolysis of p220, the high-molecular-weight component of eIF-4F (1); this cleavage is the only discernable covalent change to eIF-4F after poliovirus infection and is believed to be the cause for inactivation of eIF-4F (4). However, translation of cellular mRNAs is reduced only to ~30% of control levels. Thus, a significant number of cellular mRNAs are translated even when eIF-4F is proteolyzed (1). An intriguing possibility is that this translation occurs in some cases by internal ribosome binding.

In summary, translation of poliovirus is accomplished by internal binding of ribosomes to the mRNA via the host cellular machinery. This mode of translation initiation is independent of eIF-4F and may provide a powerful mechanism for translational control.

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#### LITERATURE CITED

- Bonneau, A.-M., and N. Sonenberg. 1987. Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. *J. Virol.* **61**:986-991.
- Both, G. W., A. K. Banerjee, and A. J. Shatkin. 1975. Methylation-dependent translation of viral messenger RNAs *in vitro*. *Proc. Natl. Acad. Sci. USA* **72**:1189-1193.
- Ehrenfeld, E. 1984. Picornavirus inhibition of host cell protein synthesis, p. 177-221. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*. Plenum Press, New York.
- Etchison, D., S. C. Milburn, I. Edery, N. Sonenberg, and J. W. B. Hershey. 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eukaryotic initiation factor 3 and a cap-binding protein complex. *J. Biol. Chem.* **257**:14806-14810.
- Jang, S. K., H.-G. Krausslich, 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.
- Kozak, M. 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. *Microbiol. Rev.* **47**:1-45.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Langridge, J., P. Langridge, and P. L. Bergquist. 1980. Extraction of nucleic acids from agarose gels. *Anal. Biochem.* **103**:264-271.
- 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.
- 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.
11. **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.
  12. **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.
  13. **Sarkar, G., J. Pelletier, R. Bassel-Duby, A. Jayasuriya, B. N. Fields, and N. Sonenberg.** 1985. Identification of a new polypeptide coded by reovirus gene S1. *J. Virol.* **54**:720–725.
  14. **Sonenberg, N.** 1987. Regulation of translation by poliovirus. *Adv. Virus Res.* **33**:175–204.