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THE 5′-UNTRANSLATED REGION OF PICORNAVIRAL GENOMES

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I. INTRODUCTION

Picornaviruses are small naked icosahedral viruses with a single-stranded RNA genome of positive polarity (Rueckert, 1985; Koch and

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Koch, 1985). According to current taxonomy (Cooper *et al.*, 1978; Matthews, 1982), the family includes four genera: *Enterovirus* (polioviruses, coxsackieviruses, echoviruses, and other enteroviruses), *Rhinovirus*, *Cardiovirus* [encephalomyocarditis virus (EMCV), mengovirus, Theiler's murine encephalomyelitis virus (TMEV)], and *Aphthovirus* [foot-and-mouth disease viruses (FMDV)]. There are also some as yet unclassified picornaviruses [e.g., hepatitis A virus (HAV)], which should certainly be assessed as a separate genus.

Studies on the molecular biology of picornaviruses might be divided into two periods: those before and after the first sequencing of the poliovirus genome. This milestone event was accomplished in the laboratories of E. Wimmer (Kitamura *et al.*, 1981) and D. Baltimore (Racaniello and Baltimore, 1981). The knowledge of the primary structure of poliovirus RNA not only solved (or helped to solve) many outstanding problems, but also confronted researchers with new puzzles. The 5′-untranslated region (5-UTR) of the viral genome was one of such unexpected problems. This segment proved to be immensely long: about 750 nucleotides, or ~10% of the genome length. There were also other unusual features (e.g., multiple AUG triplets preceding the single open reading frame (ORF) that encodes the viral polyprotein). A question arose: "What useful purpose could this giant noncoding genomic segment serve?"

In this chapter I attempt to answer this question. As we shall see, the picornaviral 5-UTRs are not only involved in such essential events as the synthesis of viral proteins and RNAs (which could be expected to some extent, although some of the underlying mechanisms appeared to be quite a surprise), but also may determine diverse biological phenotypes, from the plaque size or thermosensitivity of reproduction to attenuation of neurovirulence. Furthermore, a close inspection of the 5-UTR structure unravels certain hidden facets of the evolution of the picornaviral genome. Finally, the conclusions drawn from the experiments with the picornaviral 5-UTRs provide important clues for understanding the functional capabilities of the eukaryotic ribosomes.

II. Primary Structure

A. Size and Some Gross Features

As mentioned in Section I, the initial sequencing of the poliovirus RNA revealed the presence of a long 5-UTR, about 750 nucleotides in length (Kitamura *et al.*, 1981; Racaniello and Baltimore, 1981). Subsequent analyses showed that the 5-UTR size was remarkably constant

TABLE I

SOME FEATURES OF PICORNAVIRAL 5-UTRs[a,b]

Virus	Size (nucleotides)	Poly(C)	Number of AUGs	Initiator AUG context
Enterovirus				
Polioviruses (1–3)	742–747	–	6–8	A(C,U)(A,U)AUGG
CAV (9 and 21)	711–743	–	6–8	(A,C)AAAUGG
CBV (1, 3, and 4)	740–743	–	6–7	AAAAUGG
SVDV	742	–	10–11	AAAAUGG
Enterovirus 70	726	–	11	AUAAUGG
BEV	819	–	7	ACAAUGG
Rhinovirus				
HRV (1B, 2, 14, and 89)	610–628	–	11–13	A(U,C)CAUGG
Cardiovirus				
EMCV and mengovirus	758–842	+	5–10	AA(G,U)AUGG
TMEV (BeAn, DA, and GDVII)	1064–1068	–	8–10	A(A,C)UAUGG
Aphthovirus				
FMDV (A_{10} and O_1K)	1167–1194	+	8–11	(A,C)(A,U)(C,U)AUGA
Unclassified				
HAV	726–734	–	10	AUAAUGG

[a] References: Polioviruses: Kitamura *et al.* (1981), Racaniello and Baltimore (1981), Nomoto *et al.* (1982), Stanway *et al.* (1983, 1984a), Toyoda *et al.* (1984), Cann *et al.* (1984), Hughes *et al.* (1986), La Monica *et al.* (1986), Pevear *et al.* (1990), CAV: Hughes *et al.* (1989), K. H. Chang *et al.* (1989); CBV: Tracy *et al.* (1985), Iizuka *et al.* (1987), Jenkins *et al.* (1987), Lindberg *et al.* (1987); SVDV: Inoue *et al.* (1989), Seechurn *et al.* (1990); enterovirus 70: N. Takeda (unpublished observations, communicated by A. C. Palmenberg); BEV: Earle *et al.* (1988); HRV: Stanway *et al.* (1984b), Callahan *et al.* (1985), Skern *et al.* (1985), Duechler *et al.* (1987), Hughes *et al.* (1988); EMCV and mengovirus: Vartapetian *et al.* (1983), Palmenberg *et al.* (1984), A. C. Palmenberg (personal communication), Cohen *et al.* (1988), Bae *et al.* (1989, 1990); TMEV: Pevear *et al.* (1987, 1988), Ohara *et al.* (1988); FMDV: Forss *et al.* (1984), Newton *et al.* (1985), Robertson *et al.* (1985), Clarke *et al.* (1987); HAV: Najarian *et al.* (1985), Cohen *et al.* (1987a,b), Paul *et al.* (1987), Jansen *et al.* (1988), Ross *et al.* (1989).

[b] Abbreviations: CAV, coxsackie A viruses; CBV, coxsackie B viruses; SVDV, swine vesicular disease virus; BEV, bovine enterovirus; HRV, human rhinovirus; EMCV, encephalomyocarditis virus; TMEV, Theiler's murine encephalomyelitis virus; FMDV, foot-and-mouth disease virus; HAV, hepatitis A virus.

in different polio- and coxsackie B virus strains, varying from 740 to 747 residues (Table I). There is much greater diversity, however, among other picornaviruses. Thus, rhinovirus 5-UTRs are 610–628 nucleotides long, whereas the 5′-noncoding segments in the genomes of some cardioviruses and especially of aphthoviruses are much long-

er, in some cases nearly twice as long (up to about 1200 nucleotides) (Table I).

Partly, the greater 5-UTR size in the latter group of viruses is due to the presence of a poly(C) tract. Such a homopolymeric tract was originally discovered in the EMCV RNA (Porter *et al.*, 1974), and a similar segment was found soon afterward in the genomes of some other cardioviruses and FMDV (Brown *et al.*, 1974). The length of the tract varies among populations of the given virus, usually from about 100 to 250 nucleotides in both aphtho- and cardiovirus RNAs (Brown *et al.*, 1974; Harris and Brown, 1977; Black *et al.*, 1979; Costa Giomi *et al.*, 1984), but values as high as 600 nucleotides have also been reported (Brown, 1979).

Originally, poly(C) was erroneously suggested to lie at the 3′ end of the cardio- and aphthovirus genomes [supposedly replacing the absent poly(A)]. The actual (i.e., the nearly 5′-terminal) location of poly(C) in EMCV RNA was established in our laboratory (Chumakov and Agol, 1976), followed by similar data on RNAs of mengovirus (Perez-Bercoff and Gander, 1977) and FMDV (Harris and Brown, 1976). The distance between the RNA 5′ end and the poly(C) tract is about 150 and 360–370 nucleotides in EMCV and FMDV RNAs, respectively (Rowlands *et al.*, 1978; Chumakov *et al.*, 1979; Vartapetian *et al.*, 1983; Newton *et al.*, 1985). The 3′-terminal portion of the poly(C) tract may contain intermittent inclusions of other nucleotides, mostly uridylic acid residues (Svitkin *et al.*, 1983). Remarkably, unlike conventional cardioviruses (e.g., EMCV or mengovirus), TMEV RNA lacks a poly(C) tract (Pevear *et al.*, 1987; Ohara *et al.*, 1988).

Another contribution to the length polymorphism of the picornaviral 5-UTRs is provided by insertion/deletions of different kinds (discussed in Section X).

An important feature of the picornaviral RNAs, distinguishing them from the overwhelming majority of mRNA species operating in eukaryotic cells, is the absence of a 5′-terminal cap structure. Instead, the virion RNA contains a covalently linked low-molecular-mass virus-specific polypeptide, VPg (Lee *et al.*, 1976, 1977; Flanegan *et al.*, 1977; Sangar *et al.*, 1977; Drygin *et al.*, 1979; reviewed by Wimmer, 1982; Vartapetian and Bogdanov, 1987); the bond between nucleic acid and protein moieties is represented by O^4-(5′-uridylyl)tyrosine (Ambros and Baltimore, 1978; Rothberg *et al.*, 1978; Vartapetian *et al.*, 1980). In contrast, the majority of cytoplasmic nonencapsidated (both polyribosome-associated and "free") positive strands of picornavirus-specific RNA species from the infected cells, which actually serve as templates for the viral protein synthesis, terminate with pUp (Hewlett *et al.*, 1976; Nomoto *et al.*, 1976; Fernandez-Muñoz and Darnell, 1976;

Fernandez-Muñoz and Lavi, 1977; Grubman and Bachrach, 1979). The removal of VPg from a proportion of the viral RNA molecules to generate pUp-ended mRNA species is accomplished by host cell enzymatic activity (Ambros *et al.*, 1978). This reaction readily occurs during the cell-free translation of the VPg-terminated RNA isolated from the virions (Dorner *et al.*, 1981).

B. AUGs and Open Reading Frames

As mentioned in Section I, a characteristic feature of the picornaviral 5-UTRs is the presence of multiple cryptic AUGs preceding the polyprotein reading frame (Table I). According to the "leaky scanning" hypothesis proposed by Kozak (1986c, 1989a) (see also Section IV,A), the utilization of upstream AUGs as translational initiation signals depends heavily on the surrounding nucleotide sequence and, in particular, is facilitated by a "favorable" context, for example, an A or G residue at position −3 and a G residue at position 4, the first nucleotide in the AUG being at position 1. The majority of the upstream AUGs in picornaviral 5-UTRs actually lie in an "unfavorable" environment.

Nevertheless, it could easily be seen that most of these viruses possess at least one such AUG in quite a favorable context. Are they functional? Are the corresponding upstream reading frames (URFs) utilized for the synthesis of any polypeptides? The most likely answers are "no," even though a polypeptide encoded in a URF was reported to accumulate on *in vitro* translation of FMDV RNA (Forss *et al.*, 1984). Indeed, so far there is no evidence that any of the picornaviral URFs are translated, within the virus-infected cell, into biologically relevant polypeptides. Moreover, no phenotypic changes, at least *in vitro*, were observed on mutational inactivation (by an A-to-U transversion) of all but one (the last; position 588) internal AUG in the poliovirus 5-UTR (Pelletier *et al.*, 1988a). Nor did such phenotypic changes result from the disruption of short URFs due to insertion of tetranucleotides (Trono *et al.*, 1988a).

Why then does the position of some AUGs (and URFs) in the picornaviral 5-UTRs appear to be conserved, as originally noted for the three poliovirus serotypes by Toyoda *et al.* (1984) and as could be deduced from an inspection of certain other sequences (cf. Hughes *et al.*, 1989)? One can guess that the URFs are vestiges of the coding sequences from which the 5-UTRs evolved (cf. Section X), and that the triplets had been conserved not because of their amino acid-coding potential, but rather because of the constraints imposed on the primary and secondary structures of the relevant segments of the viral

RNAs as components of the cis-acting element(s) involved in the interaction with translation initiation factors and/or ribosomes (see Section IV,B).

C. *Conservation and Divergence*

A remarkable conservation of the primary structure of the poliovirus 5-UTR was recognized just after the genomes of the three existing poliovirus serotypes had been sequenced (Toyoda *et al.*, 1984). Further accumulation of the sequencing data revealed that 5-UTRs of other enteroviruses as well as rhinoviruses share with polioviruses many identical (or nearly identical) elements, and all of them could therefore be easily aligned (cf. Rivera *et al.*, 1988; Pilipenko *et al.*, 1989a). For cases in which the RNA primary structure is not yet available, hybridization studies revealed a close relationship among the 5-UTRs of numerous enteroviruses (coxsackie A and B, echo-, and polioviruses), except echovirus 22 and enterovirus 71 (Auvinen *et al.*, 1989; Bruce *et al.*, 1989; cf. Hyypiä *et al.*, 1989). Interestingly, the 5-UTR primary structure of coxsackievirus A9 is much more closely related to that of coxsackie B viruses (84–86% similarity) than to the nucleotide sequence of another coxsackie A virus, A21 (70% similarity) (K. H. Chang *et al.*, 1989).

A significant similarity among 5-UTRs of cardio- and aphthoviruses was also detected (Pilipenko *et al.*, 1989b), although no obvious relationship between the nucleotide sequences of the 5-UTRs of entero- and rhinoviruses, on the one hand, and those of cardio- and aphthoviruses, on the other, could be demonstrated. No noticeable similarity of the hepatitis A virus 5-UTR primary structure to the structure of this region in other picornaviral genomes was reported.

Despite the high degree of intragroup conservation, a closer comparison has revealed several examples of gross rearrangements in the individual 5-UTRs. These are discussed in Section X.

III. Secondary and Tertiary Structures

It could *a priori* be expected that the 5-UTRs would contain elements involved in the replication and translation of the picornaviral genome. These putative elements could hardly be anticipated to function solely as linear entities.

Just after the very 5′-terminal nucleotide sequences of the picornaviral genomes had become available, appropriate secondary structure models were proposed. The first such structural element recog-

nized (and experimentally supported) in the poliovirus 5-UTR was a relatively stable (approximately −20 kcal/mol) 10-bp hairpin located 9 nucleotides from the 5′ end (Larsen *et al.*, 1981) (or, avoiding a subterminal bulge, a 9-bp hairpin 10 nucleotides from the end). This feature was found to be characteristic of the genomes of all entero- and rhinoviruses investigated thus far (Rivera *et al.*, 1988; K. H. Chang *et al.*, 1989). There is evidence that the hairpin is involved in a physiologically significant function (Racaniello and Meriam, 1986) (Section VIII). Secondary structure models for the very 5′-proximal segments of the 5-UTRs were also proposed for the EMCV (Vartapetian *et al.*, 1983), TMEV (Pevear *et al.*, 1988), FMDV (Harris, 1979, 1980; Newton *et al.*, 1985; Clarke *et al.*, 1987), and HAV (Paul *et al.*, 1987) genomes. Although experimental support is available only for the EMCV model (Vartapetian *et al.*, 1983), the similarity of several elements in the EMCV, TMEV, and FMDV structures strongly suggests the existence of a consensus folding (Pevear *et al.*, 1988; Pilipenko *et al.*, 1990). These elements are likely to participate in the replication of the viral genomes (see Section VIII).

So far as the internal portions of the picornaviral 5-UTRs are concerned, there have been attempts to fold appropriate segments of individual poliovirus (Evans *et al.*, 1985), FMDV (Clarke *et al.*, 1987), and HAV (Cohen *et al.*, 1987a) RNAs by using computer programs. The models thus obtained proved to be at variance with subsequent more direct data (at least in the two former cases for which such subsequent data are available). A powerful approach to RNA secondary structure modeling (especially if this structure is expected to be of functional significance) consists of deduction of a consensus folding for numerous related RNA species by taking into account not only fully conserved nucleotide sequences, but also, and in particular, compensating mutations in pairs of noncontiguous bases (cf. Fox and Woese, 1975; Noller and Woese, 1981; James *et al.*, 1988). When this approach was applied to the picornaviral 5-UTRs, no general consensus was found; however, highly conserved secondary structure models could be derived for entero- and rhinoviruses, on the one hand (Rivera *et al.*, 1988; Blinov *et al.*, 1988; Pilipenko *et al.*, 1989a), and cardio- and aphthoviruses, on the other (Pilipenko *et al.*, 1988b, 1990). The models for entero- and rhinovirus 5-UTRs proposed by Rivera *et al.*, (1988) and Pilipenko *et al.* (1989a; see also Blinov *et al.*, 1988), while sharing many secondary structure elements, differed nevertheless from one another in some significant aspects (significant because they involved certain physiologically important regions). The models proposed by Pilipenko *et al.* (1989a,b) for entero- and rhinovirus as well as cardio- and aphthovirus RNAs were supported experimentally by testing the susceptibility to

chemical modifications and to a hydrolytic attack by single-strand- and double-strand-specific nucleases; a similar folding was also deduced for poliovirus 5-UTRs by Skinner *et al.* (1989). Figures 1 and 2 present the consensus models for the two groups of picornavirus 5-UTRs.

It is perhaps appropriate to note that the internal poly(C) tract does not appear to be involved in extensive base-pairing with other portions of either EMCV (Goodchild *et al.*, 1975) or FMDV (Mellor *et al.*, 1985) RNAs.

One should note that support for the above structures came, apart from evolutionary considerations, from experiments performed with RNA molecules in salt solutions. To what extent, however, do they reflect the RNA folding inside the infected cell? It is difficult, if at all possible to obtain a complete answer to this question. Nevertheless, genetic evidence strongly favors the *in vivo* reality of at least some elements of the proposed secondary structures. Thus, the model (Fig. 1) predicts pairing between nucleotides 480 and 525 in the poliovirus type 1 RNA. In all but one sequenced entero- and rhinovirus genome the corresponding nucleotides are expected to form an A–U pair (Pilipenko *et al.*, 1989a). The only known exception is the Sabin type 1 strain, having G_{480} instead of A; obviously, the potential to form a base pair between nucleotides 480 and 525 must in this case be diminished. In the gut of those vaccinated, however, the Sabin 1 genome appears to be unstable, and mutations in one of the two nucleotides under discussion are readily selected for. Either G_{480} is mutated back to A or U_{525} is replaced by C; the potential to form a base pair between nucleotides 480 and 525 is fully restored in either case (Muzychenko *et al.*, 1991; see also Agol, 1990). Similar evidence for the biological relevance of the same secondary structure element in the poliovirus genome can be derived from the experiments described by Kuge and Nomoto (1987) and Skinner *et al.* (1989).

The flat structures just discussed should obviously be regarded as merely a first approximation to the actual spatial organization of the picornaviral 5-UTRs. Unfortunately, no reliable tools are presently available for unraveling the tertiary structure of long RNA segments. Perhaps some useful information in this respect can be obtained by fine mapping of psorlen cross-links [e.g., exploiting the recently developed technique (Ericson and Wollenzien, 1988)], but, to our knowledge, only very long-range (i.e., visible under the electron microscope) cross-links in poliovirus RNA have been investigated thus far (Currey *et al.*, 1986).

An independent way of approaching the tertiary structure may consist of a search for evolutionary conserved potentials to form pseudoknotlike structures (cf. James *et al.*, 1988; Haselman *et al.*, 1989;

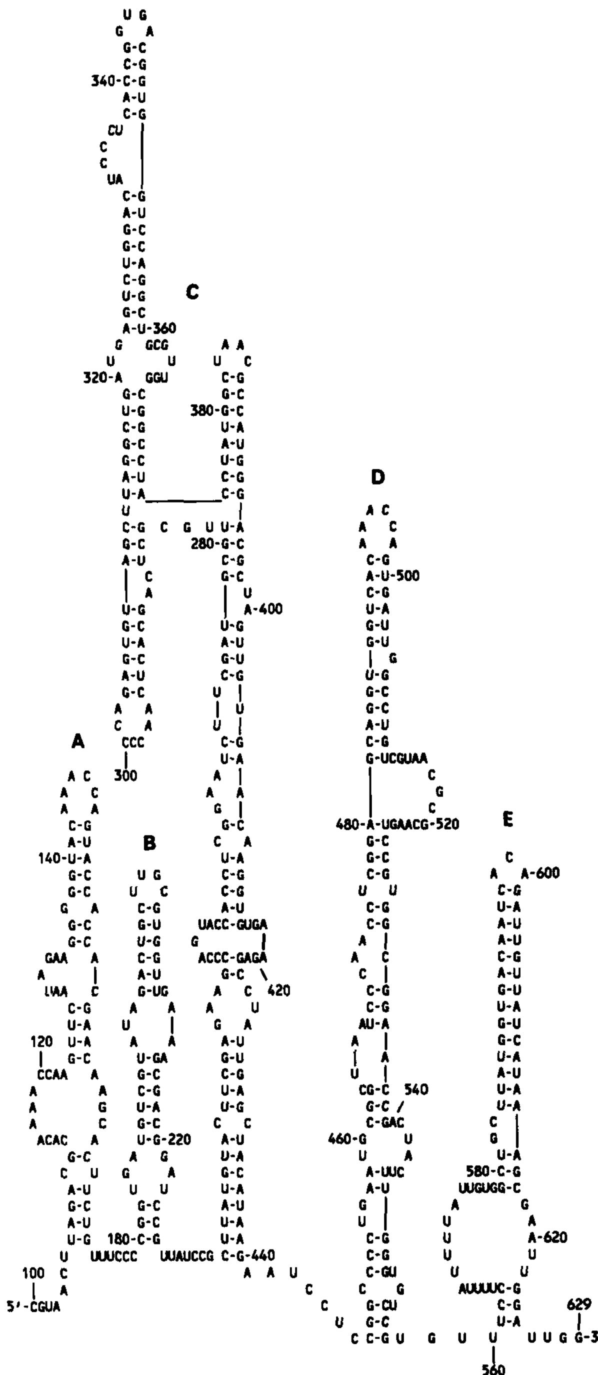
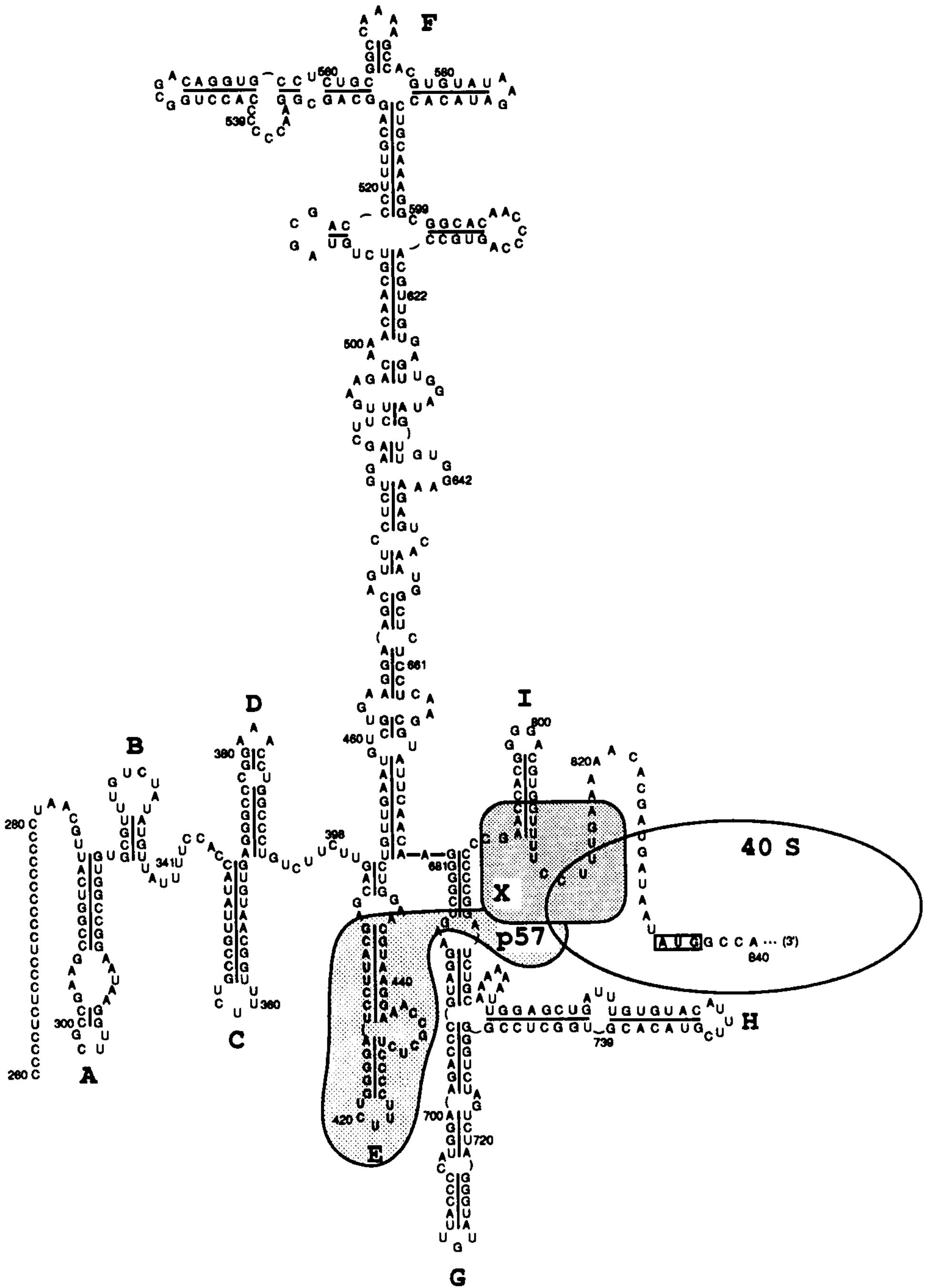


Fig. 1. A secondary structure model for the internal region of the 5-UTR of poliovirus type 1 (Pilipenko *et al.*, 1989a, 1991). This is a consensus model for all entero- and rhinoviruses.


A
B
C
D
E
F
G
H
I
X
p57
40 S
AUG
(3')
260
280
300
341
360
380
398
420
440
460
500
520
539
560
580
599
622
642
661
681
700
720
739
800
820
840

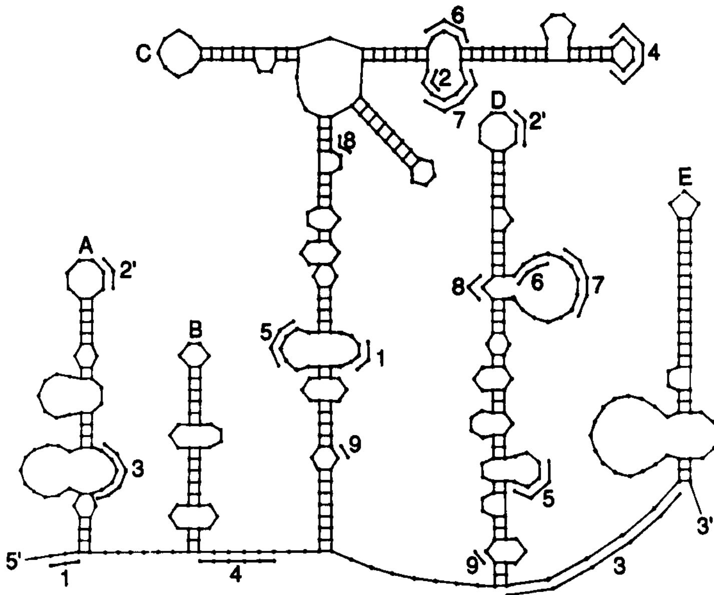


FIG. 3. Elements of the tertiary structure of picornaviral 5-UTRs. Pairs of nucleotide stretches potentially able to form conserved tertiary bonds in the entero- and rhinovirus 5-UTRs are assigned identical numbers (Pilipenko *et al.*, 1991).

Woese and Gutell, 1989). In fact, several conserved sites potentially capable of ensuring such interdomain, or other long-range, interactions in entero- and rhinovirus 5-UTRs can be envisioned (Pilipenko *et al.*, 1991) (Fig. 3). In certain cases the real possibility of such interactions *in vivo* could be supported indirectly by the observation that they tend to be maintained by coupled mutations of noncontiguous nucleotides (Muzychenko *et al.*, 1991; see also Agol, 1990). Thus, a theoretically possible pseudoknot may involve nucleotide 398 in one secondary structure domain and nucleotide 481 in another domain of the 5-UTR of poliovirus type 2. In the Sabin type 2 vaccine strain these two nucleotides might be expected to form a U_{398}–A_{481} base pair. However, among mutant Sabin 2 strains isolated from vaccine-associated cases of paralytic poliomyelitis, one can often find genomes with the two coincidentally changed bases, C_{398} and G_{481}; the potential to

FIG. 2. A consensus secondary structure model for the EMCV 5-UTR downstream of the poly(C) tract. This is essentially the model predicted by Pilipenko *et al.* (1989b) with the very terminal structures added by Jang and Wimmer (1990). Putative binding sites for polypeptides p57 and X as well as for the 40 S ribosome subunit are shown (see text for details). (From Jang and Wimmer, 1990.)

form a tertiary base pair was evidently maintained. In FMDV 5-UTRs the potential to form pseudoknots has also been identified, and similar structures can be generated in the genomes of three FMDV strains (Clarke *et al.*, 1987). The problem with these pseudoknots, however, is that they are not compatible with the predicted secondary structure of the appropriate RNA segment.

Although no direct experimental evidence is available, in light of the above considerations, the structure of picornaviral 5-UTRs is likely to be more or less condensed and maintained by specific tertiary interactions between secondary structure domains. In a sense it may be reminiscent of globular proteins. If such a viewpoint is valid, it should have obvious functional implications, and thus elucidation of the spatial (i.e., three-dimensional) organization of picornaviral 5-UTRs should be of paramount significance.

IV. CIS-ACTING TRANSLATIONAL CONTROL ELEMENT(S)

It was originally suggested by Jacobson and Baltimore (1968) that mRNA species, operating in eukaryotic cells, are functionally monocistronic, by virtue of having only a single translation initiation site. Although we are now aware of numerous exceptions to this rule, it could be accepted as an extremely useful first approximation. This biological regularity was very aptly explained by the scanning hypothesis of translation initiation proposed by Kozak (1978, 1981). The hypothesis states that a Met–$tRNA_i^{Met}$-bearing 40 S ribosomal subunit aided by specific initiation factors recognizes the capped 5′ end of an mRNA molecule and moves along it until the first AUG is encountered. Here, a 60 S subunit is recruited to form a complete ribosome, and the polypeptide chain synthesis is initiated. Accordingly, two major types of translational cis-acting elements were first considered: the cap structure and the initiator AUG.

The adaptation of the hypothesis to the newly accumulating data has led to its modification, which takes into account that the first (i.e., 5′-proximal) AUG might not necessarily be the initiator codon (Kozak, 1986c, 1989a). The modified version suggests that the ribosome scanning might be "leaky" (i.e., able to skip an AUG). Whether or not the AUG is recognized as the initiator codon largely depends on its "context" (i.e., the surrounding sequences), the optimal context being (CC)$^{A}_{G}$CCAUGG (Kozak, 1984a, 1986a) [a more extended version of the context consensus is (GCC)GCC$^{A}_{G}$CCAUGG (see Kozak, 1987a, 1987c, 1989a)]. Upstream AUGs, depending on the context, may to some extent interfere with the "correct" initiation due to "false starts," distracting ribosomes from their proper destination (Kozak, 1984b; Liu *et*

al., 1984; Hunt, 1985), but such a situation, in eukaryotic mRNAs, was considered to be "breaking the rule" (Kozak, 1989a). Moreover, there is a possibility that, on completing the translation of an ORF, the ribosome (or its smaller subunit) does not dissociate from the template, but rather continues scanning and reinitiates a new polypeptide at the next AUG; in such cases the relative efficiencies of the usage of the upstream and downstream initiation codons depends not only on their contexts, but also on other peculiarities of the given template (e.g., whether the respective ORFs are overlapping or not, how far they are located from each other and from the 5′ end, and some other conditions) (Kozak, 1984b, 1986c, 1987b, 1989b; Johansen *et al.*, 1984; Peabody and Berg, 1986; Peabody *et al.*, 1986; van Duijn *et al.*, 1988; Dabrowski and Alwine, 1988; Williams and Lamb, 1989). Thus, the upstream AUGs (and the reading frames they open) may affect the activity of the genuine initiator codons in different ways.

While testing the scanning hypothesis, one more kind of regulatory cis element was recognized in the 5-UTRs, namely, stable secondary structures. Such structures may hamper either the initial interaction of the 40 S ribosomal subunit with the mRNA (if the structured RNA element is located near the template 5′ end) (Kozak, 1989c) or the scanning process itself (Pelletier and Sonenberg, 1985; Kozak, 1986b, 1989c; Sedman *et al.*, 1990). On the other hand, unstructured regions of the template, even if they precede a stable hairpin, may facilitate initiation (cf. Kozak, 1988). Finally, a eukaryotic analog of the Shine–Dalgarno element (i.e., an oligopyrimidine sequence, just upstream of the initiator AUG, which is complementary to a segment of the 18 S ribosomal RNA) was also proposed to promote proper ribosome binding to the template (Hagenbüche *et al.*, 1978). Its significance, however, is far from certain (cf. Laz *et al.*, 1987). Thus, several, but not too many, general cis-acting signals are known to control the efficiency of translation initiation on a eukaryotic template.

As mentioned in Section II,A, picornaviral RNAs lack a cap structure. Although the idea that the terminal protein VPg might be a functional substitute for the cap was considered soon after the discovery of this protein (cf. Perez-Bercoff and Gander, 1978), it has found no experimental support and has been abandoned. Moreover, the picornaviral RNA species, serving as translational templates, appear to be not only uncapped, but also lacking VPg (see Section II,A). In fact, the realization that picornaviral templates lack a 5′-terminal cap structure was one of the first, if not the first, hint that these templates may use some noncanonical mode of translation initiation.[1]

[1]It has been reported recently that capping of the picornaviral 5-UTR may result in severe inhibition, rather than stimulation, of the template activity of an appropriate mRNA construct transfected into HeLa cells (Macejak *et al.*, 1990).

Multiple AUGs that precede the genuine initiator codon in picornavirus RNAs (Section II,B) do not appear to be directly involved in polypeptide synthesis (see also Section VI). Thus, they cannot be regarded as codons, and are unlikely to constitute, as such, any translational control signals.

What do we know about other possible cis-acting translational control elements?

A. Sequences Surrounding the Initiator Codon

When the sequences surrounding the genuine initiator codon opening the polyprotein reading frames in the picornaviral genomes are inspected, one finds that quite a favorable context (AXXAUGG) exists here in the overwhelming majority of the picornaviral genera (Table I). The exceptions to this rule are of obvious interest. First, the sequence that opens the HAV polyprotein ORF has an A residue at position 4. One may wonder whether this has anything to do with the notoriously weak template activity of the HAV RNA *in vitro* and/or the very slow reproduction of this virus within the infected cell. Second, an A at position 4 may be accompanied by a pyrimidine at position −3 in the segment surrounding the first AUG of the polyprotein-coding sequence of several FMDV serotypes (see also Robertson *et al.*, 1985; Sanger *et al.*, 1987). This may reflect the fact that only a portion of ribosomes actually initiate translation at the first AUG of the FMDV polyprotein reading frame; the others, on the other hand, appear to ignore this triplet and continue scanning until they encounter the next AUG (which, by the way, is in the frame with the previous one and is located in a favorable context). Accordingly, two sites of translation initiation could be demonstrated to operate on the FMDV genome, and the choice between these site appears to depend, at least in part, on the context of the respective AUGs (Sangar *et al.*, 1987). Third, a pyrimidine (C) occupies position −3 (and a "good" G occupies position 4) in the vicinity of the coxsackievirus A21 initiator triplet; by the way, this RNA contains at least two more upstream AUGs having a context of similar "strength" (Hughes *et al.*, 1989). The physiological significance of this "abnormality" is not immediately apparent, because the template activity of the appropriate RNA remains to be evaluated.

An oligopyrimidine tract preceding the polyprotein reading frame is present in RNAs of different picornaviruses. In aphtho- and cardiovirus 5-UTRs such a tract is situated close to the initiator codon (Beck *et al.*, 1983; Forss *et al.*, 1984; Sangar *et al.*, 1987), and there is some evidence that it is actually involved in the initiation of transla-

tion, inasmuch as its mutational alteration (in EMCV 5-UTR) might result in inefficient protein synthesis (Jang and Wimmer, 1990). The situation in entero- and rhinovirus genomes is somewhat different: Here, a conserved oligopyrimidine sequence could be found in the 560–570 region (i.e., well apart from the genuine initiator triplet). Nevertheless, there is a possibility that this oligopyrimidine tract may be involved in the interaction with ribosomes.

B. Internal Positive Control Element

1. In Poliovirus

It was against the above background that we first observed that mutations in the middle of the poliovirus 5-UTR (at positions 472–480) strongly affected the efficiency of cell-free translation initiation on poliovirus RNA [the experiments were carried out with the genomes of neurovirulent type 1 and type 3 poliovirus strains, their attenuated (Sabin) derivatives, and a neurovirulent revertant of the latter, using two *in vitro* translation systems, extracts of Krebs-2 cells and rabbit reticulocyte lysates] (Svitkin *et al.*, 1985). Since position 472 does not coincide with any internal AUGs and a mutation diminishing the efficiency of initiation should decrease, rather than increase, the stability of the appropriate secondary structure element [corresponding evidence was published later (Blinov *et al.*, 1988; Pilipenko *et al.*, 1989a)], we suggested "that a sequence around position 472 is involved, directly or indirectly, in interactions with ribosomes or initiation factors, and that the strength of this interaction may be altered by mutations at position 472" (in the poliovirus type 3 RNA) (Svitkin *et al.*, 1985). It should be noted that at that time the possibility, though unlikely, could not be rigorously ruled out that mutations other than in the region 472–480 were actually responsible for the alterations of the template activity of RNAs from attenuated poliovirus strains. However, unambiguous proof of the validity of our original assignment was provided recently by investigations of appropriately engineered templates (Svitkin *et al.*, 1990).

The suggestion that a cis-acting translational control element of a novel type exists in the middle of the poliovirus 5-UTR was supported by subsequent studies by different laboratories. First, different kinds of deletions and insertions within an extended internal segment of the poliovirus 5-UTR were shown to affect translation initiation *in vitro* (Pelletier *et al.*, 1988b,c; Trono *et al.*, 1988a; Bienkowska-Szewczyk and Ehrenfeld, 1988) and *in vivo* (Pelletier *et al.*, 1988c; Trono *et al.*, 1988b) or to change phenotypic properties (e.g., the plaque size or the

efficiency of reproduction at an elevated temperature) (Kuge and Nomoto, 1987; Trono *et al.*, 1988a; Dildine and Semler, 1989), most likely due to translation deficiencies. These studies are discussed in greater detail below.

Second, and most importantly, it was directly demonstrated that an internal portion of the poliovirus 5-UTR is involved in the cap-independent internal ribosome binding. The elegant idea of Pelletier and Sonenberg (1988) was to insert poliovirus 5-UTR into a chimeric mRNA template between two cistrons encoding readily detectable polypeptides. The efficient expression of the second cistron under conditions in which the first one was made "silent" would mean that the intercistronic poliovirus 5-UTR does secure the internal binding of ribosomes.

Specifically, several sets of plasmids were constructed. All of these sets included, among others, two monocistronic plasmids, corresponding to each of the two relevant genes, and two bicistronic plasmids, with or without intercistronically placed poliovirus 5-UTR. In one such set the first and second cistrons were represented by the thymidine kinase (*TK*) gene of herpes simplex virus type 1 and the bacterial chloramphenicol acetyltransferase (*CAT*) gene, respectively (Pelletier and Sonenberg, 1988). On transfection of tissue culture cells with a specific plasmid, relevant 5′-capped mono- or bicistronic mRNA species should be synthesized, and their translation productions, if formed, can readily be identified.

A key experiment was performed in poliovirus-infected cells (Pelletier and Sonenberg, 1988). It is well known (see Section V,B) that the canonical cap-dependent initiation is severely impaired in such cells. When poliovirus-infected cells were transfected with the above series of plasmids, the only active plasmid was that encoding the bicistronic template with the intercistronically placed poliovirus 5-UTR; the only product directed by this template was the CAT protein encoded in the second cistron (although in mock-infected cells the same plasmid directed predominantly the accumulation of the TK protein). Thus, the inactivation of the cap-dependent mechanism of translation initiation by poliovirus infection rendered the first cistron inactive, and the expression of the second cistron could hardly be attributed to the ribosomes coming from the template 5′ end. Rather, this expression should be due to the turning-on of a novel mechanism, most likely involving internal ribosome binding mediated by the intercistronic poliovirus-derived 5-UTR. The selective translation of the second cistron of the bicistronic template could be demonstrated even in the mock-infected cells, provided that the transfected cells were incubated under hypertonic conditions [it has long been known that translation initiation on poliovirus RNA, as opposed to cellular mRNAs, is much

more resistant to hypertonic salt concentrations (Nuss *et al.*, 1975)]. This experiment should be interpreted in the same way as the previous one.

Translation of bicistronic mRNAs *in vitro* lent additional support to the notion on the ability of poliovirus 5-UTR to ensure internal initiation of translation. The first cistron in the bicistronic templates used was represented either by the *TK* gene again (Pelletier and Sonenberg, 1988) or by the σS gene of reovirus type 2 (Pelletier and Sonenberg, 1989), whereas the *CAT* gene served as the second cistron in both types of constructs. In reticulocyte lysates the second cistron of the bicistronic template could be expressed, provided it had been juxtaposed to the poliovirus 5-UTR. Moreover, it continued to be expressed even when the translation of the first cistron had been abrogated by the insertion of a stable hairpin structure in its own 5-UTR (Pelletier and Sonenberg, 1988). Clear-cut results were also obtained in extracts from poliovirus-infected HeLa cells, in which the bicistronic template with the intercistronic poliovirus 5-UTR directed the synthesis of only the second gene (*CAT*) product (Pelletier and Sonenberg, 1989). The results obtained in the same study with extracts from uninfected HeLa cells were also fully compatible with the theory on the ability of poliovirus 5-UTR to direct internal ribosome binding.

Now that this theory is supported with reasonable certainty, we can attempt to deduce, from the available data, the approximate borders of the translational cis-acting element involved. To this end, it would seem logical to assume that the sequences, whose removal or mutational alterations do not appear to affect significantly the function of the element, are located outside its body. Such an approach, simple as it is, is not devoid of pitfalls. The nucleotide sequence of the element, while forming a complex three-dimensional entity, is expected to be (and actually is), in a sense, discontinuous; some of its internal portions may be (and perhaps are) of no serious functional significance. Therefore, the finding of a silent portion of the 5-UTR does not necessarily mean that it lies beyond the borders of the cis element. There is another ambiguity. The element could be considered to be composed of the essential core, whose absence results in the complete invalidation of the internal initiation mechanism and dispensable, yet functionally significant, surrounding sequences. In practice it is not always easy to judge whether a particular genomic alteration totally inactivates the element's function, affects it significantly, or leaves it unchanged. Taking into account these limitations, let us consider the experimental data accumulated thus far. First, we shall attempt to define the location of the element as a whole; then the possible functional significance of its individual components is discussed.

What can be said about the element's 3′ (downstream) border? It

would be not too surprising if the positive translational control element were located close to the initiator codon at position 743. But this is not the case. Surprisingly, Kuge and Nomoto (1987) discovered that some large deletions within the downstream portion of the poliovirus 5-UTR are fully compatible with the virus viability. Specifically, the removal of nucleotides 630–726, 622–726, or 600–726 from the poliovirus type 1 (Sabin) genome does not alter such *in vitro* phenotypic properties of the virus as the plaque size or the time course of virus growth and development of cytopathic changes (see also Trono *et al.*, 1988a). More extended deletions, 570–726 or 564–726, also produce viable progeny, but the mutant viruses exhibit a small-plaque (sp) phenotype due to their retarded growth rate (see also Iizuka *et al.*, 1989). Assuming that phenotypic changes in this series of mutants are largely due to the altered function of the cis-acting translation control element (and we believe that this is a likely assumption), it could be concluded that the downstream border of this element should map between positions 564 and 600. The border may perhaps lie even farther to the 3′ end since the 5-UTR region affected by the deletions includes repeated sequences, and therefore reformation of nearly normal structural features may be feasible in some of the deletion mutants (Pilipenko *et al.*, 1990) (see also Section X).

Another approach to the boundaries' mapping, which does not require viability of the deleted or otherwise modified genomes, consists of evaluation in their *in vitro* translational template activity. A difficulty inherent in the interpretation of the results thus obtained is due to the dependence of the outcome of the experiments on the particular cell-free system used and, sometimes, on the incubation conditions as well. An even more serious problem concerns the need of unambiguous differentiation between the internal and 5′-terminal initiations of translation, because some deletions may concurrently inactivate the former and active the latter mode.

To circumvent the latter problem, some *in vitro* translation experiments were performed in extracts from poliovirus-infected HeLa cells in which the cap-dependent initiation mode was invalidated (see Section V,B); both monocistronic (Pelletier *et al.*, 1988b) and bicistronic (Pelletier and Sonenberg, 1988) templates with a reporter gene preceded by a segment of the poliovirus type 2-derived 5-UTR were used. In agreement with the aforementioned data of Kuge and Nomoto (1987), the removal of nucleotides 632–732 did not affect the template activity, whereas the template lacking nucleotides 462–732 (or 382–732) was completely devoid, under the experimental conditions used, of the capacity to direct the synthesis of polypeptides encoded in the downstream cistron. These data placed the 3′ border of the cis element between positions 462 and 632.

So far as the 5′ border of the element is concerned, the deletion of the first 32 or 79 nucleotides from the 5-UTR was without any significant effect on the cap-independent internal ribosome binding in experiments with bicistronic templates and extracts from poliovirus-infected HeLa cells (Pelletier *et al.*, 1988b; Pelletier and Sonenberg, 1988). Dispensability, for the cap-independent internal initiation of translation, of the very proximal segment of the poliovirus 5-UTR follows also from results obtained in experimental systems for which unambiguous conclusions could not be drawn so readily, such as translation of templates with partially deleted poliovirus 5-UTR in extracts from uninfected HeLa cells (Pelletier *et al.*, 1988b; Pelletier and Sonenberg, 1988) and Krebs-2 cells (Pestova *et al.*, 1989) as well as in reticulocyte lysates supplemented with initiation factors from HeLa cells (Bienkowska-Szewczyk and Ehrenfeld, 1988). On the other hand, the removal of 139 or 319 nucleotides caused either quite small (30–40%) or marked (3- to 5-fold), depending on the construct, inhibition of the downstream gene expression (Pelletier *et al.*, 1988b; Pelletier and Sonenberg, 1988).

To sum up, we can conclude that the cis-acting element ensuring the cap-independent ribosome binding is contained within a segment with coordinates of approximately 140 to 600–630. If we accept that the cis-acting element under discussion is hardly functioning as merely a linear nucleotide sequence, then the secondary structure domains A–E (Fig. 1) should be regarded as likely components of this element.

An intriguing question concerns the functional significance of individual domains or even smaller structural entities of the whole cis-acting element. Although there is no complete answer to this question, some tentative and partial judgments could perhaps be made on the basis of the experimental data collected.

As already mentioned, the removal of about 140 5′-terminal nucleotides somewhat decreased the template activity of the poliovirus 5-UTR (Pelletier *et al.*, 1988b; Pelletier and Sonenberg, 1988). This observation may be interpreted to mean that domain A is involved in the translation initiation. Furthermore, Jackson *et al.* (1990; R. J. Jackson, personal communication) showed that nucleotides approximately between positions 70 and 160 are required in order that the translation of poliovirus RNA in reticulocyte lysates be stimulated by the initiation factors from HeLa cells (the initiation factor requirements for picornaviral RNA translation are considered in Section V,B). Nevertheless, the appropriate structure does not appear to be essential for the internal ribosome binding.

Also nonessential is domain B, although it is most likely involved in translation control. Short insertions/deletions at position 220 (note that different authors designate the positions of the same insertions

slightly differently) led to sp, ts, or even lethal phenotypes (depending on the nature of the insert), and these alterations can be traced to the impairment of the cap-independent internal initiation of translation (Kuge and Nomoto, 1987; Trono *et al.*, 1988a,b; Dildine and Semler, 1989). Significantly, large-plaque revertants of a small-plaque mutant with an insertion at position 220 were demonstrated to acquire two second-site mutations, one, invariably, at position 186 and the other, affecting nucleotides, at either 480 or 525 (Kuge and Nomoto, 1987). The first of these second-site mutations (at position 186) affected a nucleotide which is expected to interact with the region surrounding mutated position 220 (Pilipenko *et al.*, 1991),[2] suggesting the involvement of a secondary structure element. In line with such reasoning, a *ts* $^{+}$ pseudorevertant of a mutant with a 4-nucleotide deletion at position 220 was shown to acquire an additional 41-nucleotide deletion (altogether, positions 184 to 228 were lost) (Dildine and Semler, 1989), again suggesting that a (structured) RNA segment around positions 180–220 must be somehow involved in the translational control.

One more, albeit indirect, piece of evidence for the participation of domain B in a significant physiological function came from experiments with engineered recombinants between poliovirus and coxsackievirus genomes (Semler *et al.*, 1986). In one such recombinant a segment of poliovirus type 1 RNA with coordinates 220–627 was replaced by the homologous segment derived from coxsackievirus B3 RNA. The recombinant was viable, but exhibited a strong ts phenotype (although both its parents were *ts* $^{+}$). It seems likely that the defect was primarily due to some translational impairment. Moreover, this impairment resulted most probably from structural alterations at the 5′-proximal junction between the polio- and coxsackievirus RNAs (i.e., near position 220). Indeed, a *ts* $^{+}$ pseudorevertant of the recombinant acquired a deletion just downstream of this junction, and a related poliovirus–coxsackievirus recombinant, but with a more 5′-proximally located junction (at position 66), that is, having a "normal" sequence around base 220, exhibited a ts $^{+}$ phenotype (Johnson and Semler, 1988).

Although important, domain B is not essential. Its dispensability is unambiguously demonstrated by the aforementioned fact that a 45-bp delection encompassing nucleotides 184–228 is compatible not merely

[2]So far as the 480 and 525 mutations in the revertant genomes are concerned, some yet undefined long-rang nucleotide interactions cannot definitely be ruled out. However, these remote second-site mutations may have a simpler explanation. They could independently enhance the translation efficiency by increasing the stability of an important secondary structure element (see Section III), compensating, thereby, the impaired template activity of the original mutant with an insertion at position 220.

with the viability of a virus, but also with its wild-type (wt) phenotype (Dildine and Semler, 1989).

There is ample evidence that domain C (approximate coordinates 236–443) is involved in promoting the cap-independent internal initiation of translation. Thus, oligonucleotide insertions at position 267, 322, or 388 resulted in either sp and ts mutant phenotype or unviable genome, in both cases the deficiency being due to a translational impairment (Kuge and Nomoto, 1987; Trono *et al.*, 1988a,b). Importantly, the locations of physiologically significant mutations appear to affect diverse subdomains of the large domain (cf. Fig. 1), suggesting multiple intra- and/or interdomain interactions. A particular example of such an interdomain bonding (between nucleotides 398 and 481 in the poliovirus type 2 RNA) has already been discussed in Section III.

Somewhat more confusing is the question of whether domain C is essential for the cap-independent translation initiation or whether it performs merely an auxiliary role. On the one hand, the lethal phenotype of some insertion mutants mentioned above [e.g., at position 322 (Trono *et al.*, 1988a)] did testify to the indispensability of the relevant structure. On the other hand, however, the complete removal of this domain did not seem to abrogate the cap-independent initiation of translation, at least in certain cell-free systems (Bienkowska-Szewczyk and Ehrenfeld, 1988; Pestova *et al.*, 1989), although it should be noted that no rigorous proof was provided in these studies that the initiation on the domain C-lacking templates was actually accomplished by a 5′ terminus-independent (i.e., internal) mode. Perhaps the strongest evidence that the integrity of the entire domain C is not essential for the internal initiation was provided by the observation that a construct retaining only nucleotides 320–631 from poliovirus 5-UTR (and hence no intact domain C) still proved to be capable of ensuring cap-independent internal ribosome binding (Pelletier *et al.*, 1988b; Pelletier and Sonenberg, 1988). Thus, the apparent conflict of the data concerning the essentiality of domain C perhaps reflects not an entirely uncommon situation in which certain damages to a biological structure are lethal, while its complete removal is not. Another possible reason for the discrepancy is that two sets of data came from the *in vivo* and *in vitro* experiments, respectively.

An interesting locus within the cis element corresponds to a highly conserved 7-nucleotide "linker" between domains C and D (cf. Fig. 1; positions 444–450 in the poliovirus type 3 RNA). Insertion of an oligonucleotide into this linker rendered the mutant virus unviable (Trono *et al.*, 1988a). This could suggest that the linker may interact with either a trans-acting factor or a distant nucleotide sequence.

The fourth domain (D; positions 451–559 in poliovirus type 3 RNA)

appears to perform a key role; its partial or complete destruction proved to be incompatible with the ability of the cis element to direct internal ribosome binding both *in vivo* (Kuge and Nomoto, 1987; Trono *et al.*, 1988b; see also Dildine and Semler, 1989) and *in vitro* (Pelletier *et al.*, 1988b; Trono *et al.*, 1988a; Pestova *et al.*, 1989). Even point mutations within this region could modulate the efficiency of translation initiation (Svitkin *et al.*, 1985, 1988). As discussed in Section III, there is good evidence that maintenance of the secondary structure is important for the function of this domain, sometimes perhaps even more important than maintenance of the primary structure. It should be added, however, that Jackson *et al.* (1990) have reported that the deletion, from the poliovirus 5-UTR inserted between two reporter genes, of the 5′ end-adjacent nucleotides up to position 539 did not abolish the expression of the second cistron in reticulocyte lysates. Such a deletion should certainly destroy domain D. This contradiction may, again, reflect the basic differences between the requirements for the internal initiation *in vivo* and *in vitro*.

There is a highly conserved 21-nucleotide stretch which encompasses a portion of the 3′ branch of domain D as well as a linker separating domains D and E (coordinate 543–563). This stretch may perform an essential function, as first suggested by Kuge and Nomoto (1987) on the basis of the assessment of the viability of poliovirus deletion mutants. This theory was further supported by experiments with modified poliovirus (Iizuka *et al.*, 1989) and coxsackievirus B1 (Iizuka *et al.*, 1990; A. Nomoto and N. Iizuka, personal communication) 5-UTRs. Moreover, these experiments showed directly that mutations within this stretch did affect the translation efficiency. Curiously, the sequence with coordinates 539–563 in the poliovirus type 1 RNA exhibits a striking complementarity to sequence 1301–1320 in human 28 S rRNA (Iizuka *et al.*, 1989); the significance of this observation remains entirely obscure.

As far as domain E itself (approximate coordinates 582–620) is concerned, its possible significance has already been briefly discussed when we considered the 3′ border of the entire cis-acting element. It may be added that mutations within this domain, particularly those that affected a conserved AUG located in its 5′ branch, diminished the translation efficiency (Pelletier *et al.*, 1988a; N. Sonenberg, personal communication). Moreover, Bienkowska-Szewczyk and Ehrenfeld (1988) inferred from their *in vitro* experiments with truncated templates that the corresponding sequence would be essential for the internal initiation translation. Nevertheless, the fact that the removal of nucleotides 564–726 (i.e., deletion of the entire domain E) from poliovirus RNA or the corresponding segment from coxsackievirus B1

RNA did not abrogate the virus viability (but only retarded its growth) (Kuge and Nomoto, 1987; Iizuka *et al.*, 1989, 1990) indicates, perhaps, unambiguously that this domain is not essential for the virus-specific translation. Some additional information about the significance of its structural details is presented in Section V,C, and speculations about its specific role are considered in Section X.

2. In Encephalomyocarditis Virus

Simultaneously with the just described work on the poliovirus 5-UTR, similar, but independent, studies were carried out on the EMCV 5-UTR. Though seemingly identical, the relevant experiments on polio and cardiovirus 5-UTRs should not be regarded as a mere repetition of one another. It should be borne in mind that the structures of the corresponding 5-UTRs appear to be dramatically different (see Figs. 1 and 2). Just as the mechanisms of the host cell protein synthesis in poliovirus- and cardiovirus-infected cells are essentially not the same (reviewed by Ehrenfeld, 1984; Sonenberg, 1987), the modes of translation initiation might also be different. But this is not the case.

The conclusion that could be drawn from the experiments involving both template truncation (Shih *et al.*, 1987) and construction of bi- or even tricistronic templates with segments of the EMCV 5-UTR inserted between two reporter genes (Jang *et al.*, 1988, 1989) was very similar to that just discussed with regard to poliovirus: Inside the cardiovirus 5-UTR there is a cis-acting element controlling the initiation of the viral polyprotein synthesis; this element appears to be responsible for the cap-independent internal binding of ribosomes. This conclusion appears all the more substantiated after experiments with the engineered templates were performed in poliovirus-infected cells (Jang *et al.*, 1989) known to fail to initiate polypeptide synthesis in a cap-dependent mode (the cardiovirus infection itself does not bring about any apparent alterations to the cap-dependent translation machinery) (reviewed by Ehrenfeld, 1984; see also Mosenkis *et al.*, 1985).

What have we learned about the borders of the cis-acting element involved? The 5′ border should certainly be placed downstream of the poly(C) tract, since the removal of this tract, together with the adjoining upstream sequence (so-called short, or S, segment), does not significantly alter the translation efficiency of the EMCV RNA to any significant degree (Chumakov *et al.*, 1979; Shih *et al.*, 1987). Moreover, a 5′-truncated segment of the EMCV 5-UTR containing about 480 (of 833) 5-UTR nucleotides confers a high template activity to the engineered templates (Parks *et al.*, 1986; Kräusslich *et al.*, 1987; see also Elroy-Stein *et al.*, 1989). More detailed studies on EMCV RNA templates that were annealed with complementary oligodeoxyribonucleo-

tides or were 5′-truncated (Shih *et al.*, 1987) as well as on constructs containing partially deleted versions of EMCV 5-UTR (Jang *et al.*, 1988; Jang and Wimmer, 1990), while yielding somewhat deviating results, nevertheless permitted placement of the 5′ border of the essential core of the cis element somewhere downstream of position 402, that is, approximately 430 nucleotides before the initiator AUG (position 834). In any case deletions up to nucleotide 421 completely abolished the translational activity of appropriate constructs (Jang and Wimmer, 1990). Thus, the 5′ border of the control element coincides with a conserved hairpin–loop structure shown in Fig. 2. There is convincing experimental evidence that the secondary structure of this locus is no less important than its primary structure (Jang and Wimmer, 1990). It should be noted that nonessential, though significant, portions of the control element could be revealed in a more upstream segment of the EMCV 5-UTR (e.g., around positions 373–392) (Jang and Wimmer, 1990).

Exploiting the same technique of deletion of the EMCV 5-UTR placed between cistrons of a bicistronic mRNA construct, Jang and Wimmer (1990) demonstrated that the 3′ border of the translational cis element was located around position 810, that is, very close to the initiator codon that opens the polyprotein reading frame. Interesting, this border coincides with a conserved oligopyrimidine region.

Thus, cardioviruses have in common with enteroviruses an extended cis-acting control element within their 5-UTRs which ensures internal binding of ribosomes, but the primary and secondary structures as well as the locations of these elements relative to the true initiator codon are quite different in the two groups of picornaviruses.

3. In Other Picornaviruses

The nature of the cis-acting elements responsible for the ribosome binding to internal segments of the 5-UTRs of other picornaviruses has, to our knowledge, not been thoroughly investigated. Experiments with 5′-truncated 5-UTRs of rhinovirus 14 pointed to an essential role for a sequence encompassed by positions 546–621 (AlSaadi *et al.*, 1989), or perhaps to domain E and possibly a portion of domain D (Fig. 1). Moreover, a striking conservation of the primary and secondary structures also of more upstream domains (see Section III) is, in our opinion, a strong argument for the notion that entero- and rhinovirus 5-UTRs should share their major functional properties with those of poliovirus. By the same token, the TMEV and aphthovirus 5-UTRs should work similarly to that of EMCV. But there are no analogies with the HAV genome, and this system therefore needs special scrutiny.

C. A Negative Control Element

As discussed in the preceding section, the 5-UTRs of picornaviral genomes *can* and *do* promote the initiation of polyprotein synthesis through the internal cap-independent binding of ribosomes to a cis-acting control element. But what about the canonical (i.e., 5′ end-dependent) initiation of translation on these templates? Is it possible, in principle, and, if not, does it mean that a distinct negative cis-acting control element is an integral part of the picornaviral 5-UTRs?

One may argue that the ability to promote translation initiation by a cap-dependent mechanism would make no sense for picornaviral templates primarily because they simply lack a cap structure. However, it could not be ruled out in theory that portion of the VPg-devoid viral RNA molecules could be capped in the infected cell. Another theoretical argument against the possible involvement of the cap-dependent initiation system in the polyprotein synthesis is the inactivation of this system on poliovirus infection. Again, counterarguments can be put forward. First, the putative cap-dependent initiation could be imagined to occur at the very early stages of infection, prior to the inactivation of the cap-dependent factors. Second, there are examples of enterovirus infection in which no appreciable shut-off of the cellular protein synthesis could be registered due to viral mutations (Cooper, 1977; Bernstein *et al.*, 1985). Third, the inactivation of the cap-dependent machinery does not appear to take place during cardiovirus infection at all (Mosenkis *et al.*, 1985).

Therefore, the questions posed in the beginning of this section should be approached experimentally. As already mentioned, the transfection of cultured cells with capped poliovirus RNA yielded less (rather than more) viral proteins compared with the uncapped species (Macejak *et al.*, 1990). The capping of transcripts containing the entire poliovirus 5-UTR did not appreciably affect, as compared with the uncapped templates, the efficiency of the expression of the adjacent reporter gene in extracts from either mock- or poliovirus-infected HeLa cells (Pelletier *et al.*, 1988b). These facts should mean that the cap-dependent initiation of translation, if it occurs at all, could be responsible for only a negligible part of the overall protein synthesis directed by the intact poliovirus RNA. On deletions of specific segments of the 5-UTR, however, the cap dependence of translation increased markedly; it was especially pronounced with extensively truncated templates (e.g., those lacking the first 631 nucleotides); such truncated RNAs, however, could not serve as active templates in extracts from poliovirus-infected cells (Pelletier *et al.*, 1988b), in which the cap-dependent translation initiation system is inactivated (see Sec-

tion V,B). These observations may be interpreted by postulating the existence of a negative cis-acting control element or, in other words, an obstacle to ribosome scanning from the template 5′ end to the initiator codon.

This negative element could conveniently be studied in reticulocyte lysates, where, as shown by several groups (Shih *et al.*, 1978; Brown and Ehrenfeld, 1979; Dorner *et al.*, 1984; Svitkin *et al.*, 1985; Phillips and Emmert, 1986), the efficiency of polyprotein synthesis on poliovirus template is rather low due to the deficiency in an important trans factor. The diminished ability of this cell-free system to promote cap-independent internal initiation of translation was an obvious advantage for studying the efficiency of the cap-dependent 5′ terminus-dependent event.

An internally deleted template, having only the first 70 nucleotides of the poliovirus 5-UTR, was quite competent in promoting the synthesis of a reporter protein in reticulocyte lysates, whereas the presence of nucleotides corresponding to positions 70–381 was clearly inhibitory (Pelletier *et al.*, 1988c). These data are in full agreement with a related study by Howell *et al.* (1990), who joined portions of the poliovirus 5-UTR to a reporter gene (the *NS* gene of the influenza virus) and investigated the ability of the constructed templates to promote translation initiation at specific AUGs. The results showed that the template with the very 5′-terminal 66 nucleotides of the poliovirus 5-UTR serving as a leader was quite active, whereas the first 390 nucleotides or so presented a real obstacle for ribosome scanning.

Remarkably, the inhibitory cis-acting element resides in the region of poliovirus 5-UTR (nucleotides 70–380) that precedes the positive element, and this appears to make sense. The negative element may well represent a tool to prevent unnecessary ribosome scanning along certain important portions of the 5-UTR. Such scanning might result in some adverse effects, for example, in the trapping of ribosomes or in the unwinding, and thus inactivation, of the positive control element.

On the other hand, similar experiments with mRNA constructs containing portions of the 5-UTR of human rhinovirus 14 suggested that the appropriate negative element was located more downstream, namely, within, or close to, positions 491–546 (AlSaadi *et al.*, 1989). It remains to be seen whether or not this difference reflects important peculiarities of the mechanisms of expression of entero- and rhinovirus genomes, respectively.

Although not studied in detail, the efficient translation of 5′-truncated RNAs of EMCV (Parks *et al.*, 1986) and FMDV (Clarke and Sangar, 1988) suggests that negative translational control elements are commonly present in the picornaviral genomes. Accordingly, in

order to construct templates, on the basis of the picornaviral 5-UTRs, with a high capacity for the cap-dependent (or simply 5′ terminus dependent) initiation of translation, the negative cis-acting elements are removed (cf. Parks *et al.*, 1986; Nicklin *et al.*, 1987; Clarke and Sangar, 1988).

The molecular basis of negative element activity has yet to be defined. It could be proposed that the secondary structure of the appropriate segment of the 5-UTR is involved (Pelletier *et al.*, 1988c), but its actual contribution is unknown. In any case one should take into consideration that, in order to be inhibitory, the 5-UTR secondary structure elements should either be located very close to the 5′ end or exhibit a very high stability, with a ΔG better than −30 kcal/mol (Kozak, 1989c).

V. TRANSLATIONAL trans-ACTING FACTORS INVOLVED IN INTERACTION WITH THE cis-ELEMENT

A. *General Initiation Factor Requirements for the Translation of Eukaryotic Cellular mRNAs*

A brief overview of the trans factors participating in the initiation of polypeptide synthesis in mammalian cells in general seems to be appropriate prior to a discussion on the peculiarities of the initiation factors' involvement in the picornaviral RNA translation. This overview is, of necessity, sketchy and oversimplified. A deeper analysis of the diverse aspects of the eukaryotic initiation mechanism as well as appropriate references can be found in other reviews (Moldave, 1985; Proud, 1986; Sonenberg, 1988; Rhoads, 1988; Sonenberg and Pelletier, 1989).

There are three key participants in the initiation process: the ribosome, an mRNA template, and the aminoacylated (methionyl) initiator tRNA species, and each of these must be converted, with the aid of initiation factors, into an active form in order to perform its specific role (Fig. 4).

The primary participant in the initiation process is not the whole (80 S) ribosome, but rather its smaller (40 S) subunit. The 80 S ribosomes are normally in equilibrium with their 40 S and 60 S subunits. To prevent association of the subunits, the smaller one should bind two initiation factors: a huge multicomponent factor eIF-3 and factor eIF-4C (the 60 S subunit may perhaps also be temporarily blocked by binding the initiator factor eIF-6). The second participant, the aminoacylated initiator tRNA (Met-$tRNA_i$), should first form a ternary

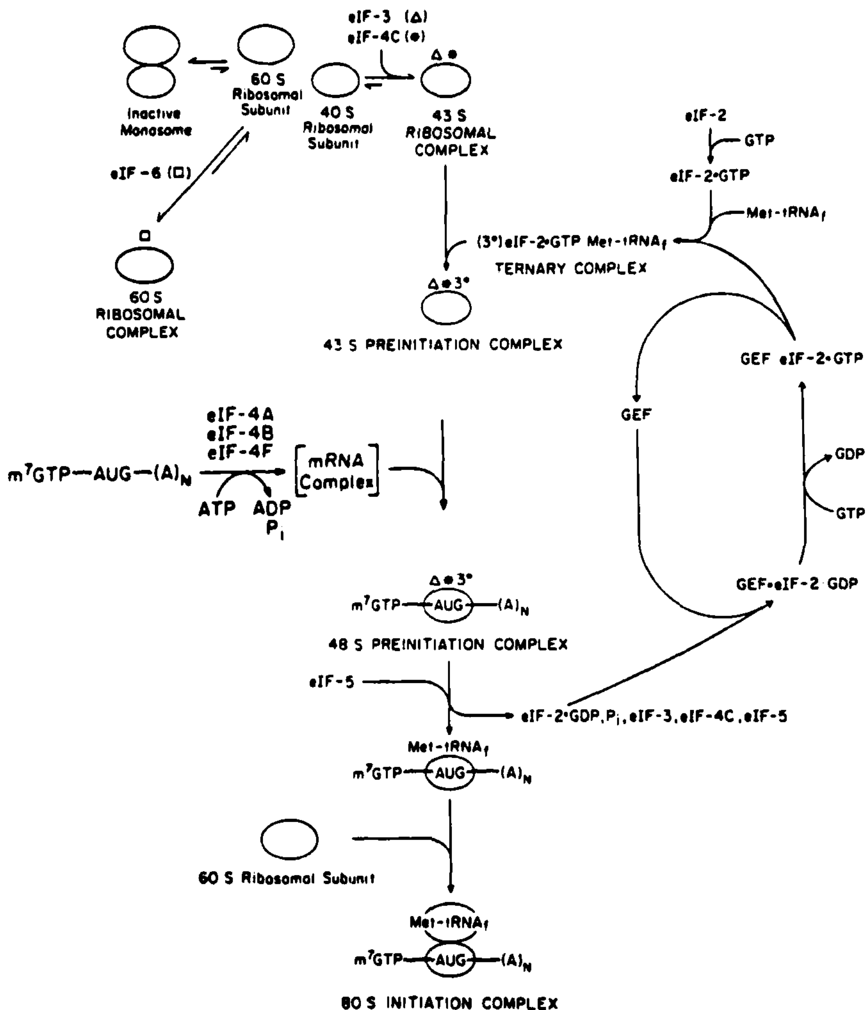


Fig. 4. Steps in the translation initiation in eukaryotes. (From Sonenberg, 1990.)

complex with factor eIF-2 and GTP. These two components, the ribosome subunit and the ternary complex, combine with one another to form a so-called 43 S initiation complex. Concurrently, the third participant, an mRNA species, which is generally capped at its 5′ end, interacts with the cap-recognizing factor, eIF-4E, which can be found in the cell both in a free form and in a complex with two other factors, eIF-4A and p220 (the complex of eIF-4E, eIF-4A, and p220 is called eIF-4F). It is believed that it is eIF-4F that actually interacts with mRNA in an ATP-dependent reaction. The next step is the interaction of the Met-tRNA$_i$-charged 40 S ribosome subunit (the 43 S initiation complex) with the mRNA template associated with eIF-4F; this reac-

tion also requires ATP and free eIF-4A as well as one more initiation factor, eIF-4B. Since eIF-4F as well as eIF-4A alone, and especially together with eIF-4B, exhibit RNA helicase properties (Ray *et al.*, 1985; Lawson *et al.*, 1989; Rozen *et al.*, 1990, quoted by Sonenberg, 1990), it was proposed that their function consists in unwinding mRNA secondary structure elements to permit ribosome "landing," which is presumed to be RNA sequence unspecific and requiring merely a "melted" segment of the template (Sonenberg, 1988; Sonenberg and Pelletier, 1989). The ribosome–template–Met-tRNA$_i$–factors complex is called a 48 S initiation complex.

At this stage a poorly understood process, the ATP-dependent movement ("scanning") of the ribosome subunit along the template ensues (Kozak, 1978, 1989a); it is not known whether the moving force for this process is provided by the ribosome itself or by initiation factors (e.g., the eIF-4A/eIF-4B helicase). The scanning continues until an AUG in a favorable context is encountered. Here, the larger ribosomal subunit (60 S) is, with high probability, recruited to form the 80 S initiation complex in a reaction which requires GTP and one more initiation factor, eIF-5, and is accompanied by the release of an eIF-2–GDP complex, as well as eIF-3, eIF-4C, eIF-5, and probably some other initiation factors. This is the end of the initiation step and the beginning of the elongation step.

Before leaving this topic, however, we should briefly mention the problem of initiation factor recycling. In fact, this problem concerns primarily factor eIF-2, because it is released from the 80 S initiation complex in a form stably bound to GDP and is therefore unable to enter a new initiation cycle. eIF-2–GDP could be converted into a reusable species through an interaction with factor eIF-2B (known also as the guanine nucleotide exchange factor, GEF) and GTP, whereby the nucleotide moiety in eIF-2–GTP is eventually regenerated. A powerful and widespread tool to regulate translation initiation consists of simply inhibiting the eIF-2 recycling by phosphorylation of its α subunit, which can be accomplished by a variety of protein kinases. The phosphorylated form of eIF-2 is, in turn, a potent inhibitor of the eIF-2B activity. The complex and physiologically important controls involving the eIF-2 system have recently been the subject of several reviews (Kaempfer, 1984; London *et al.*, 1987; Gupta *et al.*, 1987; Hershey, 1989; Sarre, 1989).

B. Initiation Factor Requirements for the Translation of Picornaviral RNAs

To comprehend the mode of initiation on picornaviral RNAs we should know, among other things, whether the initiation factor re-

quirements for the efficient translation of these templates, on the one hand, and of the common cellular mRNA species, on the other, are identical. In fact, they could not be expected to be the same in at least one respect. Due to the lack of a cap structure, picornaviral RNAs could hardly be expected to need the cap-recognizing factors eIF-4E and eIF-4F. They do not. Moreover, the p220 subunit of eIF-4F is degraded on enterovirus (Etchison *et al.*, 1982; Lee *et al.*, 1985; Buckley and Ehrenfeld, 1987), rhinovirus (Etchison and Fout, 1985), and aphthovirus (Lloyd *et al.*, 1988; Devaney *et al.*, 1988) [but not cardiovirus (Mosenkis *et al.*, 1985; Lloyd *et al.*, 1988)] infections, rendering the host cell protein-synthesizing machinery unable to efficiently initiate translation of capped mRNAs, while retaining, if not increasing, the capacity to utilize uncapped picornaviral templates (Kaufmann *et al.*, 1976; Helentjaris and Ehrenfeld, 1978; Rose *et al.*, 1978; reviewed by Ehrenfeld, 1984; Kozak, 1986d; Sonenberg, 1987). The diminished ability of cell extracts from poliovirus-infected cells to translate capped mRNA species can be restored on the addition of eIF-4F preparations (Tahara *et al.*, 1981; Grifo *et al.*, 1983; Etchison *et al.*, 1984; Edery *et al.*, 1984), supporting the theory that it is the deficiency in this factor that is responsible, at least partially, for the virus-induced shut-off of the host protein synthesis. Importantly, no evidence for poliovirus infection-induced alterations of other constituents of eIF-4F, either eIF-4A or eIF-4E, nor of another participant of the RNA–ribosome interaction, eIF-4B, was found (Helentjaris *et al.*, 1979; Duncan *et al.*, 1983; Etchison *et al.*, 1984; Lee *et al.*, 1985; Buckley and Ehrenfeld, 1986). Since poliovirus mutants unable to induce efficient inactivation of eIF-4F, being viable, grow relatively poorly (Bernstein *et al.*, 1985), it seems likely that the inhibition of the cap-dependent initiation machinery is beneficial for the virus because it eliminates competition with other templates.

The apparently diminished requirement for ATP during EMCV RNA (as opposed to capped mRNA) translation (Jackson, 1982, 1989) seems to be relevant to our discussion. This observation may suggest that relatively little work would be done by the ATP-consuming enzymes (e.g., eIF-4A/eIF-4B helicase or whatever entity is involved in the ribosome scanning) during the translation initiation on the cardiovirus template. On the other hand, a high requirement for eIF-4A was reported for the initiation of poliovirus polyprotein (Daniels-McQueen *et al.*, 1983). If there actually is a difference in the eIF-4A requirement for the poliovirus and EMCV RNA translation, it is tempting to relate it to a longer distance that a 40 S ribosome has perhaps to cover (i.e., scan) between the entry site and the initiator AUG on the former template (see Section VI). [In considering the

validity of these speculations, one should take into account, however, that Blair *et al.* (1977) reported quite the opposite results; according to their data, eIF-4A markedly stimulated the *in vitro* translation of EMCV, mengovirus, and FMDV RNAs, but not that of poliovirus.]

Inasmuch as p220 exhibits some affinity for eIF-3 (and formerly was even considered to be a component of the latter), one should not be particularly surprised if eIF-3 function were somehow modified in poliovirus-infected cells (cf. Kozak, 1986d). However, the existing data do not appear to substantiate such a possibility (Etchison *et al.*, 1984), nor is anything known about alterations in specific requirements for this factor during picornaviral RNA translation.

The next part of our discussion concerns a specific role, if any, of eIF-2 in the initiation of picornaviral polyprotein synthesis. The problem has several facets. One was set forth by R. Kaempfer and associates, who proposed that eIF-2, in addition to its canonical function in guiding Met-tRNA$_i$ to ribosomes, promotes ribosome binding to certain templates, particularly to cardiovirus (mengovirus) RNA, and probably takes part in the selection of proper AUGs as well (reviewed by Kaempfer, 1984). These proposals were based on several considerations. (1) eIF-2 was claimed to bind, with high affinity, to the neighborhood of the initiator AUG, for example, in the satellite tobacco necrosis virus RNA (which is a naturally uncapped template) (Kaempfer *et al.*, 1981) and to mengovirus RNA (Perez-Bercoff and Kaempfer, 1982); the loci of specific eIF-2 and ribosome binding appeared to overlap on these templates. (2) The addition of exogenous eIF-2 was reported to affect the competition between mRNAs with different template activities, such as α- and β-globin mRNAs or globin mRNA and mengovirus RNA, in a manner that could be interpreted to suggest that these RNAs competed for eIF-2 (Di Segni *et al.*, 1979; Rosen *et al.*, 1982). It should be noted that these ideas generally have been met with a great deal of skepticism, but we would consider it unwise to disregard them, especially in light of some recent observations. Thus, a mutational alteration in the eIF-2 β subunit led to a remarkable change in yeast's ability to choose the start codon; the mutant could now begin translation of a specific template from a UUG introduced in place of the genuine AUG (Donahue *et al.*, 1988). The ability of eIF-2 to affect the choice of initiator AUGs by the translational apparatus of rabbit reticulocytes has also been reported (Dasso *et al.*, 1990).

Another aspect of the eIF-2 system, as related to the picornavirus RNA translation, concerns regulation of its activity by phosphorylation and dephosphorylation. There is evidence that enhanced phosphorylation of the α subunit of eIF-2 accompanies poliovirus infection

(Black *et al.*, 1989; O'Neill and Racaniello, 1989), the enhancement being due to the self-phosphorylation and concomitant activation of a protein kinase induced by the accumulating poliovirus-specific double-stranded RNA (the same double-stranded RNA-stimulated kinase as that activated by interferon). Interestingly, the overall activity of this kinase in the infected cells was increased despite extensive degradation of the enzyme due to an unknown poliovirus-induced process (Black *et al.*, 1989) (one may wonder whether a poliovirus-specific protease is involved in this degradation). A similar combination of the activation and degradation of the kinase takes place during the EMCV infection too (Hovanessian *et al.*, 1987). It seems likely that the phosphorylation-triggered partial inactivation of eIF-2 in the infected cells is related to the general inhibition of protein synthesis characteristic of the final steps of the reproduction cycle (O'Neill and Racaniello, 1989).

Other reports describe, on the other hand, accumulation of an inhibitor of the dsRNA-stimulated kinase in poliovirus-infected cells (Ransone and Dasgupta, 1987, 1988) and activation of a kinase, which is also able to phosphorylate the α subunit of eIF-2, but is distinct from the double-stranded RNA-activated enzyme, during mengovirus infection (Pani *et al.*, 1986).

To sum up, we might state that the only relatively well-characterized alteration of the translation initiation factors in picornavirus-infected cells concerns the p220 subunit of eIF-4F. Inactivation of this factor provides an advantage for the virus-specific protein synthesis and furnishes additional evidence for its cap independence. Modulations of the activity of some other initiation factors, first of all eIF-2, and their possible physiological significance deserve further attention. There is no clear evidence that translation initiation on picornaviral templates exhibits an especially high specific requirement for certain initiation factors. It should be stressed, however, that thus far we have been dealing only with the generally recognized initiation factors, which have more or less characterized function(s). The possible involvement of novel factors in the translation initiation on picornaviral templates is discussed in the next section.

An additional general remark seems to be appropriate here. It is a common approach to study the initiation factor requirements for translation of picornaviral mRNAs by using an artificial host system such as HeLa cells, or even such nonhost cells as rabbit reticulocytes. Evidence is accumulating, however, that properties of the translational machinery may vary in cells of different origin or differentiation status, and that such variation may markedly affect expression of the picornaviral genomes. Thus, translation of poliovirus RNA in

human blood cells (López-Guerrero *et al.*, 1989) and neural cells (La Monica and Racaniello, 1989; Agol *et al.*, 1989) appears to be relatively restricted. A likely reason for these disparities may consist of the different availability of initiation factors (see also Section IX). The cell dependence of picornavirus translation appears to be a newly emerging and exciting area of research.

C. Search for Translational trans Factors Interacting with the 5'-Untranslated Region

As a matter of fact, any cis-acting element can work only if it interacts with something else. In principle, this "something else" may be represented by another genomic cis element or a variety of molecules of different kinds, but the first thing that comes into mind on considering the activity of an mRNA translational cis element is naturally a protein "factor." Several approaches have been pursued to identify novel factor(s) recognizing the translational cis-acting control element(s) in the middle of the picornaviral 5-UTR. Perhaps the first attempt of this kind was undertaken in our laboratory when we looked for a factor able to "sense" mutations modulating the cis element activity (Svitkin *et al.*, 1988). Our study was based on two lines of previous observations.

First, Dorner *et al.* (1984) showed that, in rabbit reticulocyte lysates, poliovirus RNA was translated predominantly from "aberrant" sites located within the polyprotein coding region, although "correct" initiation at the beginning of this region also occurred to a lesser extent. As demonstrated first by Brown and Ehrenfeld (1979) and confirmed in other laboratories (Dorner *et al.*, 1984; Svitkin *et al.*, 1985; Phillips and Emmert, 1986), crude preparations of translation initiation factors from nucleated (e.g., HeLa or Krebs-2) cells could "normalize" the pattern of products directed by poliovirus RNA in reticulocyte lysates. Thus, an "initiation correcting factor" (ICF) should be present in the active fraction from nucleated cells, whereas reticulocyte lysates appear to be ICF deficient. Second, the correct initiation was shown to be especially weak when RNA templates from attenuated poliovirus strains (known to bear point mutations within the 472–480 region of the cis-acting control element; see Section IV,B) were translated in reticulocyte lysates (Svitkin *et al.*, 1985). We wondered, therefore, whether the poor template activity of the RNAs with mutations in the 472–480 region could be explained by their diminished responsiveness to ICF. In other words, we asked whether ICF could "recognize" mutations within the cis-acting control element. The answer was "yes": the initiation of viral polyprotein synthesis was stimulated by partially

purified ICF preparations from Krebs-2 cells to a markedly greater extent when RNAs from virulent, compared with attenuated, poliovirus strains were used as templates. We inferred that ICF does interact with a region of the viral 5-UTR that encompasses mutations in the 472–480 region of poliovirus 5-UTR.

The exact nature of ICF has not been established. During several purification steps it copurified with an activity of initiation factor eIF-2, although pure preparations of eIF-2 exerted a negligible ICF effect. Our data are consistent with the assumption that ICF corresponds to a complex between translation initiation factors eIF-2 and eIF-2B (Svitkin *et al.*, 1988), but additional experiments are needed to unequivocally prove this point.

Two other (often used together) approaches to the identification of the trans factors are so-called gel retardation (mobility shift) experiments and ultraviolet (UV) cross-linking. While the meaning of the latter is more or less self-evident, the former consists of investigating the ability of different protein fractions to form complexes with RNA molecules, thereby diminishing the mobility of the latter on nondenaturing gel electrophoresis. In both cases the problem is to find a balance between two opposite requirements. On the one hand, the RNA fragment should be as short as possible, because this gives a relatively greater mobility shift on binding a protein, and, in UV cross-linking experiments, the sequence nonspecific binding will be minimized. On the other hand, the shortening of the fragment increases the chance that it will lose its original secondary and tertiary structures, and hence certain potential RNA–protein interactions would not be realized. This conflict of requirements is a real problem, especially since the length of the RNA fragment is often determined by the availability of convenient sites for restriction nucleases on appropriate plasmids, and since the secondary and especially tertiary structure of the RNA in question is rarely known.

Nothwithstanding the above reservation, experiments of this sort did demonstrate the existence of specific host cell proteins with an affinity for specific segments of poliovirus RNA. It was shown in a pioneering study by Meerovitch *et al.* (1989) that the electrophoretic mobility of a segment of poliovirus type 2 (Lansing) RNA with coordinates 559–624 (i.e., encompassing the entire domain E; see Fig. 1) was diminished on a reaction with a HeLa cell extract; this retardation was not due to a nonspecific RNA–protein interaction, since it could not be prevented by a huge excess of unrelated RNA. Two proteins, with M_r values of 52K and 100K (p52 and p100, respectively), have been identified as covalently linked to the 559–624 RNA segment after UV irradiation, but, on the basis of several controls, only the former was

considered to be specifically bound. Purified preparations of p52 stimulated *in vitro* translation of poliovirus RNA, but p52 could not be identified with any known translation initiation factors (Sonenberg and Meerovitch, 1990). Interestingly, reticulocyte lysates, notorious for their poor ability to translate poliovirus RNA (see above), appeared to be deficient with respect to p52 (Meerovitch *et al.*, 1989). Meerovitch *et al.* considered the possibility that p52 may be responsible for the ICF activity described by Svitkin *et al.* (1988).

Importantly, the A→U substitution at position 588, that is, in AUG_7 [this mutation was shown to lower the cell-free translational template activity of the viral RNA (Pelletier *et al.*, 1988a) and could be expected to destabilize the secondary structure of domain E] decreased the apparent affinity of the RNA segment to p52 (Meerovitch *et al.*, 1989). Further studies (Sonenberg and Meerovitch, 1990; N. Sonenberg, personal communication) showed that similar effects (i.e., a decrease in both template activity and p52 binding) also accompanied mutations of the two other nucleotides of AUG_7; importantly, introduction of the compensating mutations in the 3′ branch of domain E did not restore the ability to bind p52. Therefore, p52 appeared to recognize the primary, rather than secondary, structure. This notion was strongly supported by the observation that a shorter RNA segment, entirely lacking the 3′ branch of domain E and consequently devoid of the original double-stranded stem, did retain p52-binding capacity (N. Sonenberg, personal communication). It should perhaps be noted that AUG_7 lies just downstream of a conserved stretch of pyrimidine residues, and these two elements may conceivably form a common cis-acting protein-recognizing signal. However important this signal may be, it does not appear to be essential, since, as discussed in Section IV,B, deletions of the corresponding regions from the poliovirus (Kuge and Nomoto, 1987; Iizuka *et al.*, 1989) or coxsackie B1 virus (Iizuka *et al.*, 1990) 5-UTR resulted in viable progeny, although exhibiting an sp phenotype.

However, another cis-acting element, a highly conserved 21-nucleotide sequence shared by entero- and rhinoviruses, lying immediately upstream of the oligopyrimidine stretch and occupying portions of the 3′ branch of domain D and the linker between domains D and E (coordinates 543–563 in the poliovirus type 1 RNA), may well be essential (Kuge and Nomoto, 1987; Iizuka *et al.*, 1989, 1990) (see Section IV,B). This element appears to possess the capacity to specifically bind a 57-kDa cellular protein (A. Nomoto, personal communication). The nature of this protein and its relationship, if any, to p52, described in the preceding paragraph, remain unknown. It may be noted that, using a longer poliovirus RNA segment (coordinates 320–629), del Angel *et al.* (1989) also detected specific binding of a cellular protein

complex to a region with coordinates 510–629, that is, spanning both the cis-acting elements studies by Sonenberg's and Nomoto's groups; the presence of the α subunit of the translation initiation factor eIF-2 in this complex has been revealed.

Still another region of the enterovirus 5-UTRs, having a potential to bind specific cellular proteins, lies near the 5′ end of the cis-acting element and perhaps involves domain A or B (Fig. 1) or both. A 46-nucleotide segment with coordinates 178–224 specifically interacted with a 50K protein, which was distinct from p52 described by Meerovitch *et al.* (1989) as well as from translation initiation factors eIF-2, eIF-3, eIF-4A, and elongation factor EF-1α (Najita and Sarnow, 1990). The interaction seemed to involve the loop of domain B and was accompanied by an apparent partial melting of its stem. The RNA–protein complex was stable enough to suggest that it was held by a covalent bond, and there was evidence that the bond involved an SH group of the protein and a U_{202} residue of the loop (Najita and Sarnow, 1990). Although such a unique feature might suggest an important characteristic of the interaction, the relevant cis-acting element is, nevertheless, dispensable for the virus' viability (Dildine and Semler, 1989) (see Section IV,B).

Finally, a slightly more upstream segment (coordinates 97–182) was also shown to bind a distinct complex of cellular proteins, which seemed to include the α subunit of eIF-2 (del Angel *et al.*, 1989).

Thus, several, though not yet fully characterized, cellular proteins exhibit specific affinities for at least four seemingly separate sites within the enterovirus 5-UTR cis-acting translational control element. Although the functional significance of these numerous protein–RNA interactions remains obscure, it is tempting to speculate that at least some of them are directly involved in the internal entry of ribosomes (see also Section VI).

Two recent papers describe attempts to identify trans factors specifically interacting with the cis element of the EMCV 5-UTR (Borovjagin *et al.*, 1990; Jang and Wimmer, 1990). A cellular polypeptide of about 57–58K (p57) was shown to bind an EMCV RNA segment containing an imperfect stem–loop element with coordinates 401–550. The secondary structure of this element appeared to be more important than the nucleotide sequence for its protein-binding capacity, as judged by the effect of mutations either destroying or restoring the double-stranded stem (Jang and Wimmer, 1990). The changes in this capacity were in parallel with the changes in the template activity of the mutated RNA species in reticulocyte lysates, suggesting that p57 does participate in the internal ribosome binding.

VI. Current Views on the Mechanisms of Translation Initiation on Picornaviral RNAs

Thus, the existence of a cis-acting element in the middle of picornaviral 5-UTRs playing an essential part in the initiation of polyprotein synthesis is firmly established, its boundaries are tentatively defined, and some of the putative trans-acting factors involved are being characterized. Nevertheless, the specific functions of this element are far from being understood. Two major, and not necessarily mutually exclusive, ideas have been put forward to explain the mechanism of initiation in this unusual system.

According to the scenario proposed by Sonenberg and Pelletier (1989) for the poliovirus protein initiation (Fig. 5), some unidentified factor [e.g., host polypeptide p52 (Meerovitch *et al.*, 1989)] specifically binds to the cis-acting element inside the viral 5-UTR, and this binding somehow attracts a helicase complex composed of translation ini-

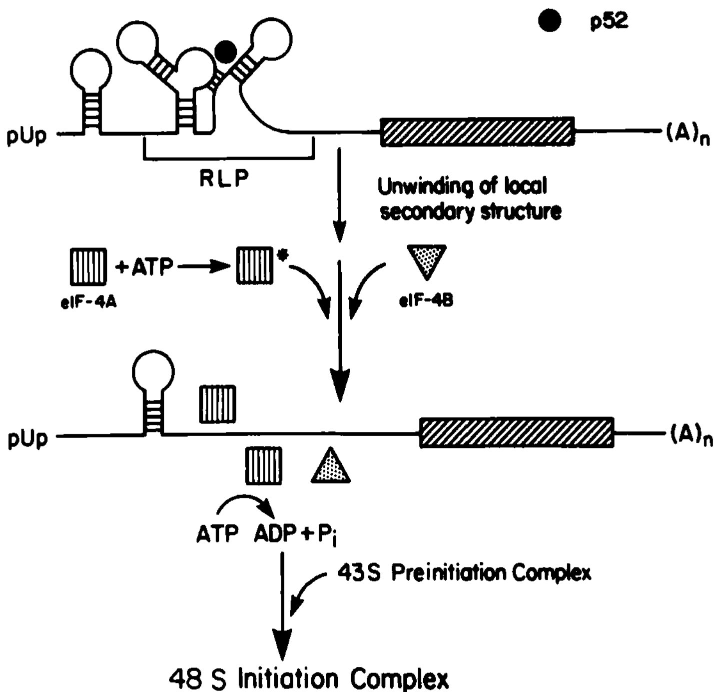


Fig. 5. A hypothetical mechanism for the internal translation initiation on the picornaviral RNAs based on the unwinding of the "ribosome landing pad" (RLP) by the helicase activity of initiation factors eIF-4A and eIF-4B. (From Sonenberg, 1990.)

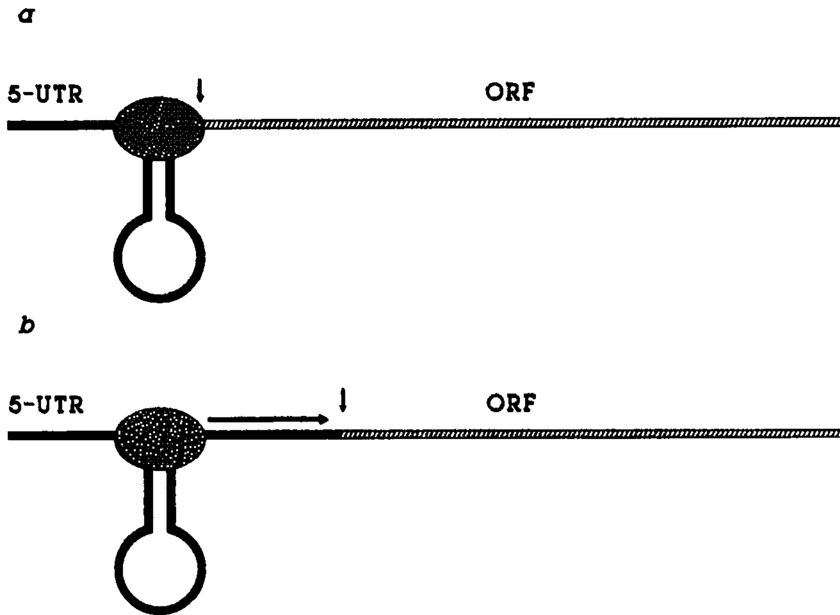


FIG. 6. Two hypothetical modes of the translation initiation on the picornaviral RNAs. (a) Direct ribosome binding to both the initiation site and cis-acting upstream regions brought into proximity by secondary and tertiary interactions. Such a mechanism is probably realized in cardio-, aphtho-, and rhinoviruses. (b) Internal ribosome binding to the cis-acting control region and subsequent ribosome scanning (horizontal arrow) toward the initiation site (vertical arrow). This mode is believed to be characteristic of poliovirus and other enteroviruses. (Modified from Howell *et al.*, 1990.)

tiation factors eIF-4A and eIF-4B. In an ATP-dependent reaction the bidirectional helicase (Rozen *et al.*, 1990, quoted by Sonenberg, 1990) unwinds a segment of the otherwise extensively structured cis element, providing, thereby, an unspecific (just single-stranded) ground for the 40S ribosomal subunit "landing." Being landed, the subunit has to obey Kozak's rules and performs a canonical walk along the 5-UTR until it encounters the correct initiator AUG that opens the polyprotein reading frame.

The other point of view, most clearly and explicitly expressed by Jackson and associates (Howell *et al.*, 1990), assumes that the cis element under discussion is involved in ribosome binding in a more direct way, serving as a framework for putting the bound ribosome (or its 40 S subunit) precisely at a key place, in some cases perhaps very closely to the initiation codon (Fig. 6). This view appears to be directly applicable to the initiation of translation on the EMCV template. Howell *et al.* (1990) compared the probabilities of initiation at the genuine AUG

(position 834), on the one hand, and at any of the three closely adjacent upstream AUGs (positions 826, 752, and 743, respectively), on the other (actually, a set of artificial templates containing a portion of EMCV 5-UTR fused to a reporter protein coding sequence was translated in reticulocyte lysates). The main conclusion emerging from these elegant experiments was that, given the presence of an intact upstream cis-acting control element, the initiation occurred almost exclusively at the correct AUG, with a striking disregard of even an AUG located less than 10 nucleotides upstream. This and the two more upstream ignored AUGs were by no means "crippled" ones, since they could ensure fairly efficient initiation, provided a significant portion of the preceding sequence was deleted. The invalidation of the cis-acting control elements (both the positive and negative ones) rendered the mode of initiation fully conforming to Kozak's rules; the most 5′-proximal AUG was not preferentially used, and the initiation was markedly stimulated on template capping.

Somewhat different results were obtained with the poliovirus RNA initiation system. It is true that different lines of evidence strongly argue for the internal entry of ribosomes and against the very possibility that ribosomes can reach the correct initiation codon (at position 743) by scanning from the 5′ end of the viral RNA (see Section IV). Nevertheless, scanning along a limited segment of poliovirus RNA adjacent to the initiator AUG seems to be substantiated by the following observations: (1) Insertion of an AUG-containing sequence downstream of position 702 resulted in the lowering of the growth potential of the mutant poliovirus (the appearance of sp phenotype) due most likely to the impairment of the viral protein synthesis; large-plaque revertants of this mutant invariably lacked the newly acquired AUG triplet (Kuge *et al.*, 1989a,b); (2) insertion of a foreign reporter gene together with its own initiator AUG at position 630 of the poliovirus 5-UTR produced a template capable of directing *in vitro* the synthesis of the reporter protein in a manner indistinguishable from that characteristic of the synthesis of genuine poliovirus proteins (Howell *et al.*, 1990); and (3) insertion of a stable secondary structure element (thought to be an "obstacle" for ribosome scanning) at position 631 of poliovirus type 2-derived 5-UTR resulted in abrogation of the expression of the following ORF (Pelletier and Sonenberg, 1988).

Thus, the cis-acting element in the poliovirus 5-UTR appears to guide ribosomes (or, perhaps more exactly, their 40 S subunits) to a specific locus over 100 nucleotides upstream of the initiator triplet, the distance they must cover by "ordinary" scanning. It seems that the point from which such scanning does start can be specified more precisely. As discussed in Section X, the poliovirus genome could well

have originated from a "rhinoviruslike" ancestor by the acquisition of an additional segment between the cis-acting translational control element and the polyprotein reading frame. In the rhinovirus type 2 genome the initiator AUG is located at position 611 (see Table I) just in the conserved secondary structure element E (Fig. 1). We propose that, in the case of entero- and rhinovirus RNAs, the ribosomes are fixed precisely near the AUG they "expect" to find at a proper place within this conserved element. If they do find it (as in rhinovirus RNA), they begin to translate; but if they do not (as in enteroviruses), they have nothing to do but to start searching for an appropriate AUG by scanning.

The now available data do not enable us to define accurately the 5-UTR locus from which the bound ribosome begins its search for an AUG triplet in other picornaviral RNAs. But in EMCV (strain R) RNA it is likely to lie between positions 834 (the genuine start codon) and 826 (the preceding AUG) (Howell *et al.*, 1990). In entero- and rhinovirus genomes the locus is expected to be located within a conserved stem–loop structure (domain E in Fig. 1), perhaps between nucleotides 611 (rhinovirus type 2 numbering; the initiator codon) and 576 (the preceding AUG).

The above considerations prompt the following specification concerning the boundaries (primarily, the 3′ boundary) of the cis-acting translational control element: One should distinguish between the intrinsic ability of this element to promote cap-independent internal ribosome binding to a template, on the one hand, and to mediate the precise positioning of the bound ribosome with respect to the initiator AUG, on the other. Experiments aimed at a more precise definition of the appropriate boundaries should take this specification into account.

One point, already briefly discussed above, deserves, perhaps, more detailed discussion. If the ribosomes were able to accurately place themselves at the correct locus of the template, how would they do this? We have argued that the picornaviral cis-acting element responsible for the internal ribosome binding should have a quasiglobular conformation due to multiple tertiary interdomain interactions (Section III). In order to secure the precise orientation of a 40 S ribosome subunit respective to the template, more than one contact point appears to be needed. We propose that such multipoint ribosome–RNA interaction is a common, and important, feature of the mechanism of translation initiation on the picornaviral RNA (cf. Jang and Wimmer, 1990). Within the framework of such a hypothesis, the abundance of both the protein-recognizing segments in the 5-UTR (Section IV) and protein factors involved (Section V) becomes easily understandable, as

does the poor predictability of the outcome of mutational alterations in the cis-acting control element.

VII. "Picornavirus-like" Properties of the 5′-Untranslated Regions in Other Viral and Cellular mRNAs

Thus, we have seen that the peculiar structure of picornaviral 5-UTRs serves a very specific function in the translation of the viral genome. However, there are few, if any, biochemical mechanisms exploited by only a single group of viruses. Moreover, the history of molecular biology teaches us that cellular counterparts could eventually be found for the overwhelming majority of mechanisms for the storage, replication, and expression of the genetic information that had been first discovered in viral systems and considered to be unique at that time. In this section I consider, above all, instances pertaining to the occurrence, among different viral and cellular templates, of such fundamental "picornavirus-like" properties as the capacity for internal ribosome binding, cap independence, and the possession of a negative cis-acting control element. In addition, I touch on the peculiarities of some mRNA species which point to other functional potentials of the long 5-UTRs with multiple AUGs. For convenience I discuss different picornavirus-like traits more or less separately; actually, they may join one another in various combinations.

A. Internal Ribosome Binding

Here, I consider only instances of what suggested to be true internal initiation, not leaky scanning or reinitiation. In this regard the case of the viral polymerase of a hepadnavirus, duck hepatitis B virus (DHBV), should perhaps be mentioned first. The enzyme is translated from a functionally bicistronic 3.3-kb template, whose 5′-proximal and -distal cistrons encode, in two different ORFs, the core protein and polymerase, respectively. The cistrons overlap one another, and the 305-nucleotides overlap region contains two AUGs in the polymerase ORF. Several lines of evidence ruled out the possibility that the synthesis of DHBV polymerase depended on the translation of the preceding cistron; in particular, the utilization of a frame-shifting mechanism was demonstrated to be highly unlikely (Schlicht *et al.*, 1989; L.-J. Chang *et al.*, 1989). The internal initiation at the first AUG of the polymerase ORF was suggested by these authors to be the favored, and, in fact, the only feasible, explanation. Similar data were obtained with another hepadnavirus, human hepatitis B virus (Jean-Jean *et al.*, 1989).

Remarkably, the same mechanism of internal ribosome binding may explain the synthesis of the polymerase encoded in a bicistronic template generated by still another retroid virus, cauliflower mosaic virus (Penswick *et al.*, 1988; Schultze *et al.*, 1990).

It was proposed that the synthesis of a polypeptide of a negative-strand RNA virus, vesicular stomatitis virus (VSV), is also initiated in a similar way (Herman, 1986). This small protein, called 7K, is encoded in the same ORF as phosphoprotein P (also known as polypeptide NS) and corresponds to the carboxy-terminal portion of the latter. Hybridization of the P protein mRNA with appropriate oligodeoxyribonucleotides (hybrid-arrest experiments) permitted blocking of the accumulation of P without affecting the appearance of 7K. Moreover, the synthesis of 7K, unlike that of P, was reported to be insensitive to cap analogs (Herman, 1986, 1987). The simplest, but perhaps not the only, way to explain both these observations is to postulate that the synthesis of 7K is initiated by internally bound ribosomes.

A peculiar case of internal initiation of translation was suggested to explain the synthesis of the so-called X protein of another negative-strand RNA virus, paramyxovirus Sendai virus (Curran and Kolakofsky, 1988). Similar to the previous example, this protein corresponds to a carboxy-terminal portion of a much larger viral polypeptide, protein P, and the former does not seem to be a proteolytic product of the latter. Accumulation of X, in *in vitro* hybrid-arrest translation experiments, also could be demonstrated under conditions excluding synthesis of P, consistent with an internal mode of initiation. The peculiarity of this initiation and its apparent distinction from the VSV case, however, is its sensitivity to cap analogs, suggesting the involvement of the template 5′ terminus (Curran and Kolakofsky, 1988).

Two interesting explanations of this apparent discrepancy, internal initiation and cap dependence, were offered by these authors. According to one explanation, eIF-4F is needed to stimulate the eIF-4A/eIF-4B-promoted unwinding of an RNA region (cf. Abramson *et al.*, 1988) to prepare the internal ribosome binding locus near the X polypeptide initiation site; the novel suggestion is that eIF-4F should reach this region only by initial binding to the 5′-terminal cap structure followed by the movement along the template (i.e., by a process similar to the ribosome scanning). The other explanation implies that ribosomes do begin their scanning along the template from the 5′ end, but, with some probability, they could skip (i.e., "jump over") an RNA segment; this hypothesis is supported by a case in which a similar ability of prokaryotic ribosomes has been well documented (Huang *et al.*, 1988). In considering these hypotheses, one should take into ac-

count, however, that the inhibition by cap analogs could be observed even with a cap-lacking template [e.g., satellite tobacco necrosis virus RNA (Smith and Clark, 1979)], indicating that the competition with a genuine cap is not the exclusive mode of the inhibitory effect of cap analogs.

The above [and some other (cf. Herman, 1989)] examples from the molecular biology of negative-strand RNA viruses are very interesting, but the conclusions on the internal initiation of translation, being derived largely from experiments of the hybrid-arrest type, need, perhaps, more rigorous proof (cf. critical comments by Kozak, 1989a). As in the picornavirus system, such proof may consist of the identification of cis-acting elements able to confer the property of internal ribosome binding to a heterologous gene of a bicistronic template.

There are several other strong candidates for the internal initiation of translation. Some of these are dealt with in the next section.

B. Cap Independence

There is a variety of positive-strand RNA viral genomes lacking a 5′-terminal cap structure and having a covalently linked protein instead; these include RNAs of calici-, como-, nepo-, poty-, and some other plant viruses. Although a detailed mechanism(s) of the translation initiation on such template is of obvious interest, little is actually known in this regard. Nevertheless, it seems appropriate to emphasize that the absence of a cap would not necessarily imply that internal ribosome binding is involved. For example, the satellite tobacco necrosis virus RNA, a naturally uncapped (and VPg-less) template (Wimmer *et al.*, 1968), appears to exploit the canonical set of translation initiation factors, eIF-4F included (Browning *et al.*, 1988); its translation could be inhibited by a cap analog (Smith and Clark, 1979), in contradistinction to the picornavirus RNAs (cf. Svitkin *et al.*, 1986).

Perhaps more intriguing is the situation in which a capped mRNA exhibits the ability to be efficiently translated in cells with inactivated cap-dependent machinery, namely, in poliovirus-infected cells (the physical and functional invalidation of the cap-dependent initiation factor eIF-4F in such cells is discussed in Section V,B). Two clear examples of such templates are late adenovirus mRNAs and a glucose-dependent heat-shock protein mRNA species.

The late adenovirus-specific mRNA species contain an ~200-bp 5-UTR, usually designated as tripartite leader [because it is composed of transcripts of three exons (see Horwitz, 1985)]. The leader has no upstream AUGs and contains a cis-acting control element ensuring translational activation late, but not early, in infection (Logan and

Shenk, 1984). This element permitted efficient translation of appropriate adenovirus mRNA species in poliovirus-infected cells (Castrillo and Carrasco, 1987; Dolph *et al.*, 1988; Jang *et al.*, 1989), suggesting that its function is independent of the cap-binding protein factors. The proposed explanation for this property is based on the determination of the leader's secondary structure; since it contains two apparently unpaired regions, it could simply bind factors eIF-4A and eIF-4B in a cap-independent manner, and the helicase activity of these factors would prepare a single-stranded landing pad of a sufficient length to secure internal binding of ribosomes (Zhang *et al.*, 1989). This explanation, which is in line with the popular view that the major function of the cap-dependent machinery is to promote melting of the mRNA secondary structure (Sonenberg *et al.*, 1982; Sonenberg, 1988), may, however, be incomplete, because it fails to take into account that the tripartite leader appears to activate translation during late, but not early, stages of the adenovirus reproduction cycle. The involvement of trans-acting factors whose concentration or activity would be changed on infection seems, therefore, quite likely. In any case the possibility that the initiation of translation of late adenovirus-specific proteins is accomplished through internal ribosome binding deserves further attention.

Another exciting system of translational control is operating during heat shock (for reviews see Lindquist, 1987; Edery *et al.*, 1987). At supraoptimal temperatures (as well as in response to some other stresses) severe inhibition of translation of the majority of cellular templates usually occurs. Partly, but not solely, this appears to be due to the inactivation of eIF-4F (Panniers *et al.*, 1985; Duncan *et al.*, 1987). Against this inhibited background, however, a class of transcripts encoding so-called heat-shock proteins is synthesized and translated with enhanced efficiencies. At least several mRNA species encoding heat-shock proteins [e.g., heat-shock protein 70 and glucose-regulated protein 78 (GRP78; also known as immunoglobulin heavy-chain binding protein)] are preferentially (compared to other cellular mRNAs) translated in poliovirus-infected cells (Muñoz *et al.*, 1984; Sarnow, 1989), once more demonstrating their independence of the eIF-4F activity.

The peculiar translational properties of the heat-shock protein templates are controlled by their 5-UTRs (McGarry and Lindquist, 1985; Lindquist, 1987), which, by the way, are relatively long (200–250 bp) (cf., e.g., Ting and Lee, 1988). Macejak *et al.* (1990), using a novel assay system (transient transfection of tissue culture cells with a specific mRNA species), showed that GRP78 5-UTR is able to confer, to a heterologous gene, translatability, and even enhanced translatability, in the poliovirus-infected cells. Moreover, the bicistronic templates, having GRP78 5-UTR as an intercistronic element, express the second

cistron under conditions prohibiting expression of the first one (P. Sarnow, personal communication). The latter experiment establishes unambiguously that these elements are able to ensure cap-independent *internal* ribosome *binding*.

One more example of the cap-independent internal ribosome binding, though not yet published in detail, concerns the translation initiation on the mRNA encoding the Antennapedia protein of *Drosophila melanogaster* involved in the differentiation of the fruit fly. This mRNA has a remarkably long (i.e., >1500-nucleotide) 5-UTR with 15 upstream AUGs (Stroeher *et al.*, 1986). When segments of this 5-UTR were introduced between cistrons of an engineered bicistronic template. independent expression of the second cistron could be demonstrated, testifying unequivocally to the internal initiation of translation (Oh *et al.*, 1990; P. Sarnow, personal communication). The positive cis-acting element responsible for such initiation appeared to adjoin closely to the initiator AUG.

It is perhaps appropriate to note that the 5-UTRs of several oncogenes are very similar, with respect to their length and sometimes to the number of upstream AUGs, to that of the Antennapedia; the mRNA encoding human c-*abl* (Bernards *et al.*, 1987) and c-*sis* (Ratner *et al.*, 1987) oncogenes could be taken as examples. mRNAs of cellular proteins other than oncogenes, for example, that of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Reynolds *et al.*, 1984), the rate-limiting enzyme of cholesterol biosynthesis, also has an apparently similar organization. It could be expected that all these templates exploit a common mechanism for translation initiation.

Thus, mRNAs for certain important cellular proteins, or even classes of such proteins, are likely to share with picornaviral and other viral RNAs the propensity for cap-independent internal ribosome binding. Physiological, and possibly evolutionary, significance of this fact has yet to be elucidated.

C. Negative cis-Acting Control Elements

Two kinds of negative translational control elements within eukaryotic 5-UTRs are often considered—secondary structures and upstream AUGs—which may function separately or together.

A remarkable example of what appears to be the regulatory element of the first kind is provided by mRNA encoding a cellular oncogene, c-*myc*. Like many other oncogene templates (cf. Kozak, 1987c), c-*myc* mRNAs of different origin possess a long (i.e., several hundred bases) 5-UTR; at least in some species, these GC-rich 5-UTRs contain no AUGs (Watt *et al.*, 1983; Bernard *et al.*, 1983; Saito *et al.*, 1983). Al-

though we are aware of no direct determinations of the secondary structure of the *myc* mRNA 5-UTR, computer modeling predicts the existence of multiple base-paired elements both between 5-UTR and coding sequences (Saito *et al.*, 1983) and within 5-UTR itself (Parkin *et al.*, 1988a). The *myc* 5-UTR contains a negative translational control element, as judged by a significant increase in the *myc* mRNA template activity on its 5′ truncation (Darveau *et al.*, 1985), and it was proposed that the activation of Myc protein synthesis (and concomitant tumorigenesis) on the translocation of a portion of the *myc* gene into a different genetic background (e.g., as in Burkitt's lymphomas) may result, at least in part, from the removal of this element (Saito *et al.*, 1983).

Remarkably, like the poliovirus negative cis element, the *myc* 5-UTR element exerts its translation inhibiting activity in reticulocyte lysates or *Xenopus* oocytes quite well, being nearly inactive in cultured mammalian cells or extracts therefrom (Parkin *et al.*, 1988a). The reason(s) for these differences is unknown, but it may be speculated that reticulocyte lysates are deficient in either the RNA duplex unwinding (if the negative effect of the cis element is due to an RNA secondary structure) or an unidentified translation initiation factor, which should interact with the cis element to override an obstacle of some other kind (Parkin *et al.*, 1988a); in any case a striking resemblance to the peculiarities of poliovirus RNA translation in reticulocyte lysates is obvious.

It can well be imagined that the negative element takes part in the physiological control of the *myc* gene expression as well. If this is true, then a mechanism for overcoming the element's inhibitory effect should exist. Evidence strongly supporting this theory was reported by Lazarus *et al.* (1988), who observed that, unlike *Xenopus* oocytes, *Xenopus* eggs or embryos could quite efficiently support translation of the *myc* mRNA.

A functionally similar negative translational control element has recently been discovered in the 5-UTR of another human oncogene, *BCR/ABL*. This GC-rich element has the potential to form a stable secondary structure, and its ability to control the efficiency of translation initiation is cell type dependent (Muller and Witte, 1989).

Inhibitory effects of secondary structure elements within 5-UTRs have also been reported for coronavirus (Soe *et al.*, 1987) and human immunodeficiency virus type 1 (Parkin *et al.*, 1988b), porcine proopiomelanocortin (Chevrier *et al.*, 1988), and bovine liver mitochondrial aldehyde dehydrogenase mRNAs (Guan and Weiner, 1989) as well as for other templates.

The involvement of another cis-acting element, upstream AUGs, in

the control of translatability of numerous mRNA species also was often suggested, though more rarely documented. A few examples are viral mRNAs of retroviruses (Hackett *et al.*, 1986; Petersen *et al.*, 1989; Hensel *et al.*, 1989), caulimoviruses (Baughman and Howell, 1988; Fütterer *et al.*, 1988, 1989), papovaviruses (Khalili *et al.*, 1987; Sedman *et al.*, 1989), parvoviruses (Ozawa *et al.*, 1988), and herpesviruses (Geballe and Mocarski, 1988); the list also includes templates encoding cellular proteins, such as a variety of oncogenes (cf. Marth *et al.*, 1988; Rao *et al.*, 1988), ornithine decarboxylase (Kahana and Nathans, 1985; Fitzgerald and Flanagan, 1989), and a nuclear protein, PET111, controlling mitochondrial translation in *Saccharomyces cerevisiae* (Strick and Fox, 1987). Detailed analysis of the nature of these controls, however, has yet to be performed. Moreover, a contribution of elements other than the upstream AUGs (e.g., stable hairpins) can be no means be excluded in some of these cases.

D. Some Specific Potentials of Long 5′-Untranslated Regions with Multiple AUGs

The examples that follow do not necessarily differ fundamentally from at least some of those already discussed; they are, however, studied in enough detail to reveal certain specific potentials of the URFs.

The GCN4 protein of *S. cerevisiae* (GCN stands for general control nonderepressible) plays a key role in the regulation of amino acid metabolism, being a transcriptional activator of many unlinked genes involved in amino acid biosynthesis. Its own expression, normally repressed, is tremendously activated on amino acid starvation. GCN4 protein synthesis is controlled at the translational level by cis-acting signals in the 5-UTR of its mRNA as well as by several trans-acting protein factors (reviewed by Hinnebusch and Mueller, 1987; Hinnebusch, 1988).

The 5-UTR of GCN4 mRNA is about 600 nucleotides long (perhaps there are several closely spaced transcription start sites) and it contains four AUGs followed by very short (i.e., only two- or three-codon) URFs (Thireos *et al.*, 1984; Hinnebusch, 1984). These URFs, together with adjoining sequences, constitute a cis-acting control element; their deletion relieves translational repression of GCN4 protein synthesis under nonstarvation conditions (Thireos *et al.*, 1984; Hinnebusch, 1984), and an ~240-bp segment of the GCN4 mRNA containing these URFs confers the GCN4-specific controls to a heterologous gene (Mueller *et al.*, 1987). Negative effects of upstream AUGs in eukaryotic mRNAs are not a surprise (see Section IV,C); in the case of GCN4 template, however, the role of such AUGs is far from trivial.

Thus, different URFs appear to have very distinct functional properties. URF4 and URF3 (numbering is from the 5′ end) are the strongest with respect to repression activity; when all other URFs were inactivated, the modified template with a single URF4 or URF3 exhibited, under nonstarvation conditions, nearly as low a translational activity as an mRNA with intact GCN4 5-UTR; the negative effect of the single URF1 was about 30 times lower (Mueller and Hinnebusch, 1986).

This differential activity could only partly be explained by the distance of the respective URF from the GCN4 start codon; nor was there any appreciable difference in the intrinsic initiation efficiencies of the first and fourth AUGs; unexpectedly, a functional difference appeared to be traced to events occurring during, or soon after, the termination of translation of short URFs (Mueller *et al.*, 1988; Miller and Hinnebusch, 1989). These authors propose that a sequence to the 3′ side of the URF4 promotes ribosome conversion into a form unable to reinitiate at the downstream, bona fide, GCN4 initiation codon. On the other hand, on completing translation of the first URF, ribosomes retain, if not increase, the ability for further scanning of the template and for the efficient reinitiation at the next AUG. The molecular basis of these striking differences in the functions of the 5′-proximal and distal GCN4 URFs remains obscure (cf Williams *et al.*, 1988).

What is known, however, is that these differences appear to be directly related to the physiological function of the cis-acting control element. While URF4 (and perhaps also URF3) is likely to ensure repression of GCN4 protein synthesis under nonstarvation conditions, the URF1 behaves as a positive control element and is primarily involved in the translational activation (derepression) on amino acid deficiencies (Mueller and Hinnebusch, 1986).

This system can work properly, however, only when aided by several trans-acting factors (Hinnebusch, 1985; Harashima and Hinnebusch, 1986). These factors include, among others, products of negative (*GCD1* gene) as well as positive (*GCN2* and *GCN3*) regulators. Under good nutrition conditions the *GCD1* gene is active and its product somehow ensures that ribosomes, on completing their working cycles (initiation, reading, and termination), first at the upstream and then at the downstream URFs, severely diminish their ability to reinitiate at the GCN4 ORF [this notion primarily stems from the observation that *GCD1* mutants efficiently synthesize the GCN4 protein constitutively (see Hinnebusch, 1985)]. Amino acid deficiency, however, stimulates certain *GCN* genes [in particular, *GCN2*, which contains a protein kinase domain (Roussou *et al.*, 1988) and a domain closely related to histidyl-tRNA synthetase (Wek *et al.*, 1989), as well as *GCN3* (Hannig and Hinnebusch, 1988)], and this stimulation in turn

results in abrogation of the activities of the negative elements (the GCD1 protein and/or the downstream URFs), accompanied by a dramatic increase in the reinitiating capacity of the ribosomes and hence in the GCN4 mRNA translatability (Tzamarias and Thireos, 1988). Hypothetical models explaining the complex interaction of the diverse cis- and trans-acting elements involved, possibly including modulation of the eIF-2 activity, have recently been put forward (Tzamarias *et al.*, 1989; Hinnebusch, 1990).

Still another kind of translational regulation based on the interaction between a 5-UTR cis-acting element and a trans-acting protein factor is exemplified by the ferritin system. Ferritin, a ubiquitous intracellular iron storage protein, is composed of multiple copies of two subunits, light (L) and heavy (H). Ferritin synthesis is controlled post-transcriptionally, with iron deficiency resulting in the decreased translatability of both L and H ferritin mRNA species (reviewed by Theil, 1987). The cis-acting iron-responsive control element was traced to relatively long (i.e., around 200-bp) 5-UTRs of these mRNAs, particularly to their highly conserved stem–loop structure just below 30 bp in length (Hentze *et al.*, 1987; Aziz and Munro, 1987; Rouault *et al.*, 1988). This structure is specifically recognized by a protein repressor, and the affinity between the cis- and trans-acting elements increases on iron deficiency (Leibold and Munro, 1988; Walden *et al.*, 1988, 1989; Rouault *et al.*, 1988, 1989; P. H. Brown *et al.*, 1989) due to the reversible reduction of a sulfhydryl group of the repressor (Haile *et al.*, 1989).

The two regulatory systems just described seem to bear no obvious resemblance to the known function of the appropriate control element within the picornaviral 5-UTRs. Nevertheless, these systems tell us what additional functional potentials are inherent in long 5-UTRs. It would be tempting to speculate that at least some of these potentials may be exploited during the picornaviral reproduction cycle as well, for example, for alterations of the relative efficiencies of the initiations at different sites of the template; it should be noted, however, that there is as yet no experimental evidence to support such a theory.

VIII. Structure of 5′-Untranslated Region and RNA Replication

The second essential function I am going to deal with is the replication of the viral genome. One should note that, although the structure of 5-UTR itself could, in principle, affect the replication process, it is the 3′, rather than 5′, end of the RNA molecule that is expected to contain essential cis-acting signals responsible for the specific tem-

plate recognition by an RNA-dependent RNA polymerase or whatever other entity is involved. However, since the 3′ end of the complementary, or negative, RNA strand serving as a template for the synthesis of the viral, or positive, strand is mirrored in the 5-UTR structure, it is convenient to say that a given 5-UTR mutation affects the synthesis of viral RNA, although the 5-UTR complement may actually be involved.

One may wonder whether the recognition signals are shared by the positive and negative RNA templates of a given virus. If this is so, some structural similarity between the 3′ termini of the opposite strands, or, in other words, some complementarity between the 5′ and 3′ termini of the same strand should exist. The 5′ and 3′ termini of picornaviral RNAs are, however, obviously unrelated, being represented by a heteropolymeric sequence and a poly(A) tract, respectively. This may indicate that the signals responsible for the positive and negative template recognition are substantially different. On the other hand, the cis-acting signals, especially those located in the positive RNA 3′ end, could well be subterminal. Indeed, there is some complementarity between the 5′ end- and poly(A)-adjacent sequences of poliovirus RNA molecules, but its functional significance remains unknown.

It should be noted that the functional analysis of the 5-UTR elements involved in replication is far from complete. The removal of over 100 5′-terminal nucleotides resulted in the loss of viability (Racaniello and Baltimore, 1981), most likely because of an RNA replication defect. Deeper insights came from more subtle interventions in the 5-UTRs structure. Racaniello and Meriam (1986), while constructing different poliovirus cDNA clones, isolated a mutant of the type 1 virus lacking nucleotide 10. In the original genome C_{10} is expected to interact with G_{34}, forming the lowest base pair in a 9-bp stem of a hairpin (Larsen *et al.*, 1981), so this stem should be truncated in the mutant RNA. The C_{10}-lacking mutant exhibited a ts and sp phenotype, was deficient in the synthesis of both positive and negative RNA strands at a nonpermissive temperature, and demonstrated somewhat retarded virus-specific protein synthesis at any temperature (Rancaniello and Meriam, 1986). Most likely, the primary lesion in the mutant reproduction was in the viral RNA replication. Several *ts*$^{+}$ pseudorevertants have been isolated from the mutant virus stock, and they invariably retained the original mutation (loss of C_{10}), but all acquired a second-site mutation, $G_{34} \rightarrow U$. Notably, this second-site mutation could be envisioned to restore the original length of the hairpin stem, if the formation of a G_9–U_{34} base pair were postulated (Racaniello and Meriam, 1986). Other independent evidence also appears to suggest an important functional role for the

secondary structure, not merely the nucleotide sequence (R. Andino, personal communication).

There is no ready answer as to the possible mechanisms of the adverse effect of the C_{10} loss as well as of the beneficial effect of the second-site mutation at position 34 on the viral genome replication. These mutations perhaps impair and restore, respectively, an important cis-acting replication signal. The nature of the compensating mutation suggests that this signal may be related to a secondary structure element. If this suggestion is correct, we would think that the signal is located in the positive, rather than negative, strand, since no restoration of the stem could be expected in the negative strand (the relevant positions are occupied by C and A residues) (Racaniello and Meriam, 1986). How a secondary structure element in the vicinity of the 5′ end of the positive RNA strand can affect the replication process remains entirely unclear. Alternatively, the effect of mutations may be attributed to alterations in the primary, rather than secondary, structure of the RNA templates (be they of positive or negative polarity or even double-stranded). In this case, also, no obvious explanation for the relationship between mutations at positions 10 and 34 could be proposed.

The involvement in the genome replication of another, more downstream, secondary structure element was also documented. There is a very convenient *Kpn*I restriction endonuclease site near position 70 of the poliovirus cDNA. This site corresponds to the second (from the 5′ end), imperfect, hairpin of the viral RNA, encompassing nucleotides 51–78 (cf. Rivera *et al.*, 1988). There is strong evidence that the secondary, rather than primary, structure of this element is important for efficient genome replication (R. Andino, personal communication). A 4-bp deletion (expected to nearly destroy the element) resulted in the loss of viral viability (Kuge and Nomoto, 1987), whereas a 4-bp insertion at the same site (leading merely to an enlargement of the loop) produced a mutant with ts and sp phenotypes as well as a severe lesion in viral RNA synthesis (Trono *et al.*, 1988a). Viruses with an incomplete reversion to the wt phenotype (i.e., partially restoring the ability to synthesize RNA and produce progeny at 37°C) could readily be selected from the mutant population; remarkably, independent clones of these "revertants" retained the original mutation (i.e., the insertion at position 70) (Andino *et al.*, 1990). Surprisingly, the suppressing mutations in the pseudorevertant genomes mapped to the sequence encoding polypeptide 3C, a viral protease. Although the suppressors appeared to be active only in cis, an analysis of the phenotypic expression of several site-directed mutations in the *3C* gene led Andino *et al.* to the conclusion that the polypeptide itself, rather than

RNA, was responsible for the suppression. If so, then one could postulate that the 3C polypeptide interacts with a hairpin in the poliovirus 5-UTR and that this interaction is involved in the viral genome replication (Andino *et al.*, 1990).

The molecular basis for this involvement is again obscure. Among likely possibilities, the protease bound to the positive RNA 5′ terminus or negative RNA 3′ terminus may promote the liberation of VPg from its precursor, or the protease moiety of an uncleaved RNA polymerase precursor promotes proper fixation of the latter on the RNA template.

Thus, there is strong evidence that about 80 5′-terminal nucleotides are involved in poliovirus RNA synthesis, but a detailed understanding of the underlying mechanisms is still lacking. Taking into account that both aforementioned hairpins are strongly conserved among entero- and rhinovirus genomes (Rivera *et al.*, 1988), we may assume that these mechanisms, whatever their nature, are also conserved in these two picornavirus genera.

We are aware of no experimental data directly demonstrating the involvement of 5′-terminal structures of cardio- and aphthovirus RNAs in the replication reactions, but remarkable conservation of certain secondary structure elements in these RNAs (see Section X) is highly suggestive of such an involvement.

IX. Phenotypic Expression of Mutations

Since picornaviral 5-UTRs take part in at least two essential steps of viral reproduction—translation and replication—one could expect that diverse mutational alterations of this segment would be phenotypically expressed as inhibiting viral growth. The retarded or otherwise impaired virus growth is generally reflected in sp, ts, or cs (cold-sensitive) phenotypes. The identification of such mutations could tell us about the *in vivo* significance of this or that RNA structural element. Especially instructive are cases in which revertants to the wt phenotype were selected and characterized. Numerous examples of such types of analysis were mentioned in the preceding sections.

Here, I intend to discuss picornaviral 5-UTR mutations that alter the virus interaction with specific target cells or organisms. As we shall see, this problem is directly related to certain facets of viral pathogenesis and cell differentiation.

Involvement of the poliovirus 5-UTR in determining the level of viral neurovirulence could first be suspected when the primary structure differences in this segment of the genomes of attenuated (Sabin) strains and their neurovirulent counterparts belonging to either type 1

(Nomoto *et al.*, 1982) or type 3 (Stanway *et al.*, 1984a) had been detected. This suspicion was greatly reinforced by the discovery that reversion to neurovirulence of the Sabin type 3 vaccine strain was often accompanied by a back mutation in position 472 (Cann *et al.*, 1984; Evans *et al.*, 1985); thus, the neurovirulent parent and revertants have a C residue at this position, whereas the attenuated strain has a U residue there. Further experiments with engineered genomes composed of segment derived from attenuated and neurovirulent strains have established unambiguously that the attenuated phenotype of the Sabin type 1 (Kawamura *et al.*, 1989), type 2 (Moss *et al.*, 1989), and type 3 (Westrop *et al.*, 1989) strains is partly due to peculiarities of their 5-UTRs, particularly the segment encompassing nucleotides 472–481 (reviewed by Almond, 1987; Nomoto and Wimmer, 1987; Racaniello, 1988; Agol, 1988).[3] Furthermore, different arbitrary alterations of this segment resulted in significant changes in the level of viral neurovirulence (Skinner *et al.*, 1989).

Several lines of evidence suggest that phenotypic expression of the attenuating mutations located within the 472–481 region of the 5-UTR is tissue specific.[4] Thus, two engineered strains differing from one another only in position 472 of their type 3-derived 5-UTR were shown to produce one log different harvests in human neuroblastoma cells (but not in HeLa cells), the attenuated strain being less proficient (La Monica and Racaniello, 1989; see also Agol *et al.*, 1989). But the neural tissue does not appear to be the only one that could "sense" attenuating mutations. Since the reversion $U_{472} \rightarrow C$ in the Sabin 3 genome is readily selected for in the gut of those vaccinated (Minor and Dunn, 1988), but not in conventional tissue culture cells, it seems likely that the intestinal virus-sensitive cells, whatever their nature, also efficiently discriminate against the U_{472}-containing poliovirus type 3 genomes. There appears to be a similar, gut tissue-specific, discrimination against G in position 480 and against A in position 481 of poliovirus type 1 and type 2 genomes, respectively (Minor and Dunn, 1988; Muzychenko *et al.*, 1991; Pollard *et al.*, 1989).

Other evidence for the host dependence of the expression of mutations in the 472–481 region of the poliovirus RNA consists of the fact

[3]These data refer to the Sabin vaccine strains and they do not mean that *any* attenuated poliovirus should obligatorily have appropriate mutations within its 5-UTR; thus, the 472–481 region of the Koprowski attenuated type 2 poliovirus strain W-2 is identical to that of the virulent Lansing strain (Pevear *et al.*, 1990).

[4]Other 5-UTR mutations (e.g., deletions of nucleotides 564–726 from the genomes of both virulent and attenuated poliovirus type 1 strains) may also lead to a decrease in the level of neurovirulence, but appropriate mutants appear to exhibit a diminished reproductive capacity in any host cells (Iizuka *et al.*, 1989).

that the appropriate mutants exhibited a ts phenotype in certain tissue cultures, being ts $^+$ in others (P. D. Minor, personal communication).

Although it was originally suggested that the attenuating point mutations within the poliovirus 5-UTR could bring about dramatic changes in the secondary structure of the relevant segment of the poliovirus genome (Evans *et al.*, 1985), it was later found that, instead, they generally resulted in certain destabilization, not gross rearrangement, of the structure characteristic of the neurovirulent counterpart (Pilipenko *et al.*, 1989a; Skinner *et al.*, 1989; Muzychenko *et al.*, 1991).

As discussed in Sections IV and V, the most likely reason for the attenuating character of these mutations is the impaired ability of the mutated RNA to initiate polyprotein synthesis (Svitkin *et al.*, 1985). A natural question then arises as to why this translation defect should be tissue specific? Although the exact answer is unknown, a possible clue is provided by the observation that translation initiation factor eIF-2 seems to be involved, directly or otherwise, in the interaction with the cis-acting control element (Svitkin *et al.*, 1988; del Angel *et al.*, 1989; see also Section V). Since the eIF-2 system is known to be subject to diverse controls (London *et al.*, 1987; Hershey, 1989), it could well be imagined that its activity is dependent on the status of cell differentiation. Therefore, the apparently extraordinary sensitivity of neural cells to attenuating mutations in the 5-UTR control element seems, in general, to be understandable and, in fact, has been predicted (Svitkin *et al.*, 1985, 1988; Agol, 1988). Moreover, it could be suggested that the ability of even wild-type poliovirus strains to cause a paralytic disease in only a minute proportion of infected nonimmune humans (Melnick, 1985) could partly be due to physiological or pathological alterations of the translation machinery in their central nervous systems (Agol, 1988, 1990).

It should be emphasized, however, that direct involvement of the eIF-2 system is likely, but not rigorously proved, and by no means is the only possible way to explain tissue specificity of the expression of attenuating mutations located within the poliovirus 5-UTR. Evidence is accumulating that other newly emerging and less characterized initiation factors, such as p52 (Meerovitch *et al.*, 1989) or p100 (T. V. Pestova, unpublished observations), could be involved in the recognition of the poliovirus 5-UTR cis-acting signals, on the one hand, and may exhibit a certain degree of cell specificity, on the other (see Section V,C).

Whatever the nature of the tissue specificity of the attenuating mutations in the poliovirus genome, the very existence of such specificity and its apparent relation to the 5-UTR structure raises the possibility of creating completely nonvirulent polioviruses by fusing the

poliovirus coding sequence with a (portion of) 5-UTR borrowed from a nonneurotropic picornavirus. Such polioviruses would prove to be very promising for live vaccines.

As to the aphtho- and cardioviruses, there is ample evidence that the poly(C) tract is somehow involved in their pathogenicity. The first hint at the possibility of such an involvement was reported by Harris and Brown (1977), who observed that selection, by passages in a tissue culture, of an attenuated FMDV strain from the virulent parent was accompanied by a marked decrease in the poly(C) length (from 170 to 100 nucleotides). Although a later study by another group failed to reveal a simple correlation between FMDV pathogenicity and poly(C) length (Costa Giomi *et al.*, 1984), this group did detect an apparent contribution of the homopolymeric tract length to the reproductive capacity of the virus in the organism (Costa Giomi *et al.*, 1988). Indeed, these authors noted that strains having a longer poly(C) tract had been selected for on chronic infection of steers, though at late stages shortening of the originally long poly(C) was also observed. Mixed infection experiments with the two FMDV isolates differing from each other with respect to the poly(C) length demonstrated that the strain with a longer poly(C) had a selective growth advantage (Costa Giomi *et al.*, 1988).

More clear-cut evidence for the involvement of poly(C) in virulence was obtained with EMCV. Nearly complete removal of the poly(C) tract (e.g., leaving only eight C residues), while not impairing its reproductive capacity in tissue culture cells (Duke and Palmenberg, 1989), rendered the virus strongly attenuated (Duke *et al.*, 1990). Such a *poly(C)*$^-$ attenuated virus turned out to be a very efficient live vaccine able to prevent superinfection of mice with lethal doses of wild-type EMCV. The molecular mechanism underlying the attenuated phenotype of the *poly(C)*$^-$ EMCV mutant has yet to be established.

Possible involvement of 5-UTR sequences other than the poly(C) tract in the control of mice neurovirulence of another cardiovirus, TMEV (which has no such tract), follows from experiments reported by Calenoff *et al.* (1990). These authors constructed a set of infectious recombinant cDNA clones composed of segments derived from the genomes of attenuated and neurovirulent strains. Although the precise mapping of the appropriate determinants has yet to be done, some indirect evidence allowed these authors to suggest that a region downstream of position 790 might be especially important.

A comparison of nucleotide sequences of several EMCV variants with different biological properties permitted the suggestion that the ability to induce interferon *in vitro* may be related to a U insertion at

position 765, which could potentially add a base pair to a secondary structure element (Bae *et al.*, 1989).

Several groups (Cohen *et al.*, 1987a,b; Jansen *et al.*, 1988; Ross *et al.*, 1989) noted that HAV strains differing in the levels of attenuation to monkeys or of adaptation to growth in tissue culture also exhibited several 5-UTR alterations [which may or may not be reflected in modifications of the RNA secondary structure (cf. Cohen *et al.*, 1987a,b)]. An actual contribution of the first 347 nucleotides to the latter property has been documented (Cohen *et al.*, 1989).

It is obvious that biological relevance should not necessarily be attributed to every 5-UTR mutation found in a picornavirus with altered behavior. There is a 1- to 3-nucleotide difference in the poly(C) length among the related genomes of diabetogenic and nondiabetogenic EMCV variants, respectively (Cohen *et al.*, 1988; Bae *et al.*, 1989, 1990). Nothing suggests, however, that this minor deviation has any physiological significance; rather, the biological difference should perhaps be attributed to an amino acid change in the VP1 site possibly involved in the interaction with β cells (Bae *et al.*, 1990). One should note that there is no general theory able to predict the biological outcome of this or that 5-UTR mutation.

X. Some Evolutionary Considerations

The data presently available do not allow us a serious discussion on the origin of picornaviral 5-UTRs. Nevertheless, an analysis of 5-UTR structure variations among different picornavirus representatives could provide insights into how some specific segments of this region could evolve. Several clues emerged from the discovery of gross rearrangements (duplications and insertions/deletions) within certain 5-UTRs (Pilipenko *et al.*, 1990).

We already know that the essential cis-acting translational control element lies far upstream (i.e., > 100 nucleotides) from the initiator AUG in the poliovirus RNA. How did this element find itself at such a remote, and unique, position? Our hypothesis is based on the existence of directly repeated sequences located in the segment preceding, and partly intruding into, the polyprotein coding region (Pilipenko *et al.*, 1990) (Fig. 7). Actually, there are two such duplications. The repeating unit of the first duplication is over 100 nucleotides long, and its two copies (S′ + L′ + C′ and S + L + C) occupy, in poliovirus type 1 Mahoney strain, positions 533–645 and 670–772, respectively (the initiator AUG starts at residue 743 and defines the upstream border of the C region). The segment separating these repeating units (positions

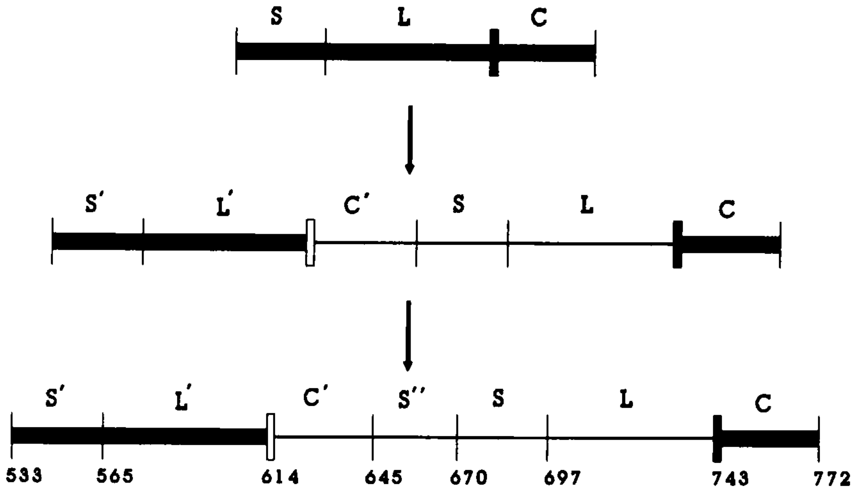


FIG. 7. A hypothetical reconstruction of the origin of the enterovirus genome. S and L, Short and long segments of the repeating unit; C, the region encoding an amino-terminal portion of the viral polyprotein; solid horizontal bars, the most conserved and functionally most important segments; solid vertical bars, actual initiator AUGs of the polyprotein reading frame; open vertical bars, mutated or otherwise inactivated initiation codons. The numbers correspond to the nucleotide positions in the poliovirus type 1 RNA. For other explanations, see text. (From Pilipenko *et al.*, 1990.)

646–669) is, in turn, a unit (S″) of another tandem duplication; the other, downstream, unit (S) of the latter corresponds to a 5′-terminal portion of the distal unit of the larger, just mentioned, repeating element. Importantly, the rhinovirus 5-UTR has only a single copy of the sequence corresponding to the poliovirus S + L + C element, even though in other respects the polio- and rhinovirus 5-UTRs are strikingly similar. On the basis of these observations, we proposed the following partial reconstruction of the poliovirus genome evolution (Fig. 7).

A predecessor of poliovirus had a rhinoviruslike genome, that is, the translational cis element (S + L) adjoined the beginning of the polyprotein reading frame (C), and it was present as a single-copy sequence. Then, a duplication of this element occurred, accompanied by the appearance of an additional functional initiation codon which, according to the Kozak's theory, could interfere with normal initiation. The adverse effect of such a development could be abolished by inactivation of the upstream AUG by a mutation (in fact, AAG is located here in the present-day poliovirus RNA). In addition the unwanted reading frame could be destroyed in other ways as well, for example, by an additional shorter duplication (e.g., by the appearance

of S″). The most important parts of this newly evolved structure corresponded to the 5′ segment of the upstream repeating unit (S′ + L′), where the cis element is located, and to the 3′ segment of the downstream unit (C), harboring the beginning of the polyprotein coding-sequence. Just these two parts of the polio genome turned out to be much more conserved as compared to the "insert" (C′ + S + L).

As discovered by Kuge and Nomoto (1987), this insert could be deleted without inflicting any apparent damage to the viral phenotype. This should not be a great surprise, since, due to the duplication, such deletions might render nearly complete restoration of the secondary structure of the essential control element (Pilipenko *et al.*, 1990). Despite the fact that this insert portion of the poliovirus genome is certainly not essential, it is present (in a more or less diverged form) in the RNAs of all enteroviruses investigated so far. This suggests that it is hardly devoid of any biological significance; perhaps it could participate in fine translational controls.

The above considerations allow us to suggest that the primary interaction of ribosomes with rhinovirus RNA should be in a sense more similar to their interaction with EMCV RNA than with seemingly more related poliovirus RNA: In the two former cases the ribosome is expected to be positioned just adjacent to the initiator triplet and ready to start translation, whereas in the latter case it should scan a distance between the landing site and the initiator triplet (cf. Section VI).

Some other rearrangements were found closer to the end of the picornaviral 5-UTRs, and are, therefore, more likely to affect replication, rather than translation, of the viral genome. Thus, a direct tandem repeat >100 nucleoties in length (positions 7–115 and 116–230) could be revealed in the genome of bovine enterovirus (BEV) (Pilipenko *et al.*, 1990). The repeating units can fold into nearly identical secondary structure elements that, in turn, are very similar to a single-copy element found by Rivera *et al.* (1988) to be conserved among different entero- and rhinovirus genomes; as already discussed in Section VIII, mutations in this conserved sequence affect replication of poliovirus RNA (Racaniello and Meriam, 1986; Andino *et al.*, 1990). It seems likely that at least the upstream repeating unit of the BEV 5-UTR could be specifically recognized by the viral genome replication machinery. The biological relevance, if any, of the downstream unit has yet to be established.

An interesting rearrangement was also detected on a comparison of the primary and secondary structures of the poly(C)-preceding segments in EMCV and FMDV 5-UTRs. These segments could form stem–loop structures (Vartapetian *et al.*, 1983; Newton *et al.*, 1985) with several conserved, among EMCV and FMDV RNAs, secondary

structure elements and an approximately 250-nucleotide insertion into the loop in the latter case (Pilipenko *et al.*, 1990). Despite this large insertion into the terminal structure, apparently involved in the genome replication, the essential recognition elements appear to be conserved in the FMDV RNA due to long-range interactions. The origin of this insertion is unknown, although a weak similarity to a region of FMDV RNA downstream from poly(C) could be revealed.

It may be noted that in the genome of another cardiovirus, TMEV, there is a long segment that appears to replace the poly(C) tract of such cardioviruses as EMCV or mengovirus (Pevear *et al.*, 1987). Although the origin of this replacement remains a mystery, it may be related to pathogenic or other host-related properties of the virus.

Thus, a variety of duplications and large insertions/deletions could be discerned in the picornaviral 5-UTRs. In addition, relatively short repeating elements 3′ from FMDV poly(C) were described previously (Clarke *et al.*, 1987). Moreover, it was speculated that the entire poliovirus genome had originated through the multiplication of short genetic elements (Gorbalenya *et al.*, 1986).

The mechanism of generation of repeats and other rearrangements is unknown, but it is most likely related to template switches postulated to occur during the replication of picornaviral RNA (Romanova *et al.*, 1986; Kirkegaard and Baltimore, 1986; Kuge *et al.*, 1986). The generation of adjacent (i.e., tandem) direct repeating elements requires a single "jump" of the nascent chain, perhaps in association with the RNA-dependent RNA polymerase, from one template to another (or from one locus of a template to another locus of the same template) (cf. Romanova *et al.*, 1986), whereas for the appearance of noncontiguous repeating elements (or the insertion of "foreign" sequences) at least two such jumps are needed. The duplication-generating jumps would obviously be facilitated if the template contained short direct repeats flanking the sequence to be duplicated.

There is another intriguing evolutionary problem. Coding sequences for picornaviral nonstructural proteins are remarkably like those of several plant virus families, especially como- and nepoviruses, suggesting their close interrelatedness (Argos *et al.*, 1984; Franssen *et al.*, 1984; Greif *et al.*, 1988). However, the 5-UTRs of true picornaviruses and picornavirus-like plant viruses, as already mentioned, hardly share any significant structural features, except for 5′-terminal VPg (instead of a cap structure). It could be speculated that the picornaviral 5-UTRs are either a relatively late acquisition, at least in comparison with the age of their coding sequences, or that this structural and functional genomic unit had, for whatever reasons, been lost at birth or during subsequent evolution of the plant viral genomes. One may

wonder whether the above distinction reflects certain intrinsic differences in the mammalian and plant translational machineries.

XI. Concluding Remarks

On reviewing the wealth of existing knowledge related to picornaviral 5-UTRs, one might perhaps be left with somewhat mixed feelings. On the one hand, the efforts of numerous research groups appear to be fairly rewarded, and the field, which was discovered only a decade ago, can undoubtedly be regarded as one of the most advanced areas of molecular virology. It gives insights not only into the mechanisms of viral reproduction and behavior, but also into the working abilities of the eukaryotic translational machinery in general. On the other hand, the understanding, in molecular terms, of the activities of the translational and replicational cis-acting elements within the picornaviral 5-UTRs is yet to come. Among the questions most urgently awaiting adequate answers, I would list the following.

1. What is the spatial organization of the 5-UTRs, and of their cis-acting regulatory elements in particular?
2. What is the nature of the protein trans factors interacting with the relevant cis elements of the 5-UTRs, and how do these two partners recognize one another?
3. What are the physiological outcomes of the interactions between the trans factors and the cis elements?
4. Why is the expression of some 5-UTR mutations tissue dependent?
5. What is the origin of the 5-UTRs?

Obviously, most of these questions could be, and actually have been, put forward even before any systematic investigation of the picornaviral 5-UTRs was initiated. Not less evident, however, is the fact that adequate answers to these questions are now within much easier reach.

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