Mutational analysis of the 5' non-coding region of human immunodeficiency virus type 1: effects of secondary structure on translation

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The first 111 nt from the 5' end of human immunodeficiency virus type 1 (HIV-1) mRNAs are shown to have a strong inhibitory effect on the translation of mRNA in *in vitro* translation extracts as well as in *Xenopus* oocytes. Mutations in the sequence of the 5' untranslated region (UTR) designed to disrupt predicted secondary structure of this region relieve the inhibition. Inhibition is restored by mutations that reconstruct the predicted secondary structure. The accessibility of the 5'-terminal cap structure was also found to be increased by some of these mutations. We conclude that secondary structure in the 5' UTR of HIV-1 mRNAs and resultant inaccessibility of the cap structure is responsible for the inhibition of translation. The implications of these findings for the understanding of the life cycle of HIV-1 are discussed.
Key words: AIDS/human immunodeficiency virus type 1/latent infection/mRNA secondary structure/translation control

Introduction

The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of the acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Popovic *et al.*, 1984). HIV-1 is a highly regulated retrovirus; replication of the virus is controlled by several *trans*-acting genes as well as *cis*-acting regulatory sequences located within the viral long terminal repeat (LTR) and elsewhere in the viral genome (Arya *et al.*, 1985; Rosen *et al.*, 1985, 1986; Sodroski *et al.*, 1985a, 1986; Knight *et al.*, 1987; Peterlin *et al.*, 1986). Two of the *trans*-acting genes, the *trans*-activator (*tat*) (Arya *et al.*, 1985; Sodroski *et al.*, 1985b; Wright *et al.*, 1986) and the anti-repression *trans*-activator (*art*) (Sodroski *et al.*, 1986; Knight *et al.*, 1987), also called *trs* (Feinberg *et al.*, 1986), are essential for virus replication. The *tat* gene acts to accelerate the rate of virus production (Arya *et al.*, 1985; Sodroski *et al.*, 1985b; Rosen *et al.*, 1986; Feinberg *et al.*, 1986), whereas the *art* gene differentially regulates the expression of virus structural and regulatory proteins (Sodroski *et al.*, 1986; Knight *et al.*,

1987; Rosen *et al.*, 1988).

The *cis*-acting sequences responsive to the *tat* gene product, the *trans*-acting responsive (TAR) region, are located immediately adjacent to the site of transcription initiation (Rosen *et al.*, 1985); the TAR sequence is therefore located at the 5' end of all viral mRNAs. The TAR sequence and flanking 3' region contain inverted repeat sequences that permit the mRNA to assume a stable secondary structure at its 5' end (Okamoto and Wong-Staal, 1986; Muesing *et al.*, 1987; Patarca *et al.*, 1987). A high degree of secondary structure at the 5' end of mRNAs has been shown to inhibit translation (for a recent review see Pelletier and Sonenberg, 1987), and therefore we considered the possibility that this structure also plays a role in regulation of translation of HIV-1 mRNAs. In the present study, we have used *in vitro* systems as well as microinjected *Xenopus* oocytes to characterize the translational effects of the 5' untranslated region (UTR) of HIV-1 mRNA at the 5' end of a heterologous mRNA.

Results

Inhibition of translation in vitro by HIV LTR sequences

To determine the effect of the inverted repeat structure at the 5' end of the HIV-1 viral mRNAs (Okamoto and Wong-Staal, 1986; Muesing *et al.*, 1987; Patarca *et al.*, 1987), a series of plasmids was constructed in which sequences derived from the HIV LTR were placed 5' to the coding sequence of the bacterial enzyme, chloramphenicol acetyltransferase (CAT). The HIV LTR-CAT sequences were placed downstream of the SP6 bacteriophage RNA polymerase promoter (Melton *et al.*, 1984). The RNA produced by transcription of these plasmids was used to program *in vitro* translation extracts. RNA was synthesized in the presence of m⁷GpppG under conditions that have been shown to result in an efficiency of capping of the nascent RNAs of >90%, as judged by chemical determination of 5' end cap structures (Pelletier and Sonenberg, 1985a).

The plasmids used to produce mRNAs are pictured in Figure 1A. The RNA produced from pSP64/CAT contains 43 nt 5' to the initiation codon for the CAT gene: nucleotides 1–6 derived from pSP64 and nucleotides 7–43 derived from the CAT gene as present in pSV2CAT (Gorman *et al.*, 1982). For plasmids pSP64/TARCAT and pSP64/TAR(+111)CAT, the 5' region of the product mRNA corresponds to nucleotides +1 to +80 and +1 to +111 of HIV mRNAs, respectively, followed by the same sequences between the *Hind*III site and the AUG codon as in pSP64/CAT. For plasmid pSP64/TAR(+111R)CAT, the first 80 nt correspond to +1 to +80, whereas the next 31 nt are complementary to nucleotides +81 to +111 of the HIV LTR (i.e. antisense).

mRNA produced from these plasmids was used to program lysates capable of *in vitro* protein synthesis. The RNA was also microinjected into *Xenopus* oocytes. The

Fig. 1. (A) SP6 plasmids used to synthesize mRNA are shown diagrammatically; for details of the constructions, see Materials and methods. SP6, promoter sequence for SP6 RNA polymerase; CAT, chloramphenicol acetyltransferase gene; TAR, *trans*-acting responsive element containing +1 to +80 of the HIV LTR. (B) Predicted secondary structure in the 5' UTR of HIV-1. An estimate of the free energy of formation of this structure, calculated using the guidelines of Tinoco *et al.* (1973), is approximately -53 kcal/mol.

Fig. 2. (A) Translation of CAT mRNAs in reticulocyte lysate. SP6-generated mRNAs were translated at a concentration of $2.5 \mu\text{g/ml}$ for 30 min at 30°C in reticulocyte lysate in the presence of $[^{35}\text{S}]$ methionine. An autoradiogram of labeled protein after SDS-PAGE is shown. Lane 1, no mRNA added; lane 2, CAT mRNA; lane 3, TARCAT mRNA; lane 4, TAR(+111)CAT mRNA; lane 5, TAR(+111R)CAT mRNA. Molecular masses of protein standards, in kd, are indicated. (B) As for (A) but in HeLa cell extract. Translations were done at 37° for 60 min. (C) Translation of CAT mRNAs in *X. laevis* oocytes. Oocytes were injected with mRNAs (10 ng) and incubated at 21°C for 10 h. Conversion of $[^{14}\text{C}]$ chloramphenicol (Cm) to its acetylated forms (AcCm) after incubation for 3 min with extracts from injected oocytes is shown. Lane numbers and designations are as in (A). For CAT (lane 2), the equivalent of 10% of an oocyte was used; for all others, the equivalent of one oocyte was used.

relative translational efficiency of each mRNA was determined by comparing the amount of CAT protein produced, either by labelling with $[^{35}\text{S}]$ methionine followed

by SDS-PAGE and fluorography, or by assaying for enzymatic activity. When using CAT assays, all measurements were made in the linear range of the assay.

Table I. Relative translational efficiencies of CAT mRNAs containing the 5' UTR of HIV-1

Translation system	mRNA			
	CAT ^a	TARCAT	TAR(+111)CAT	TAR(+111R)CAT
Reticulocyte lysate ^b	100	0.48	0.06	0.36
HeLa extract ^c	100	10	2	5
<i>Xenopus</i> oocytes ^b	100	2.5	0.24	2.5

^aThe amount of CAT protein synthesized from the CAT mRNA was set at 100%.

^bValues were determined by quantitation of acetylated and non-acetylated forms of chloramphenicol after CAT assays (see Materials and methods), and represent an average of two to three independent experiments.

^cValues estimated by comparing the intensities of the ³⁵S-labeled CAT protein by densitometric tracing after SDS-PAGE and fluorography.

Representative results from translations in reticulocyte lysate, HeLa cell extract, and microinjected *Xenopus* oocytes are shown in Figure 2, and summarized in Table I (all quantitative estimates of relative translational efficiencies were obtained by assaying for CAT activity using aliquots of the translation reactions). The results show that the presence of the 5' UTR of HIV-1 inhibits the translation of CAT mRNA in *cis*. In the reticulocyte system, nucleotides +1 and +80 from the HIV 5' UTR (TARCAT) inhibit translation of CAT ~200-fold (compare lanes 2 and 3 in Figure 2A; see Table I). The addition of nucleotides +81 to +111 from the LTR further decreases the efficiency of translation (~8-fold less compared to TARCAT; compare lanes 3 and 4 in Figure 2A). When this sequence is present in the antisense form, however, translation is as efficient as for TARCAT (compare lanes 3 and 5 in Figure 2A). Comparison of the translation efficiencies of CAT and TARCAT mRNAs in a HeLa cell extract gave similar results, but the degree of inhibition exerted by nucleotides +1 to +80 was ~10-fold and that of nucleotides +1 to +111 was 50-fold (see Figure 2B and Table I).

The mRNAs were also microinjected into *Xenopus laevis* oocytes. Figure 2C shows the conversion of chloramphenicol to its acetylated forms after incubation with extracts from injected oocytes. The CAT mRNA is translated ~40-fold more efficiently than TARCAT (compare lane 3 to 2), and 400-fold more efficiently than TAR(+111)CAT (compare lane 4 to 2). When nucleotides +81 to +111 are present in the antisense form translation is as efficient as for TARCAT (compare lane 5 to 3). The results are therefore similar to those obtained in the reticulocyte system. These observations reinforce the concept that the inhibition of TARCAT translation is an inherent property of the mRNA, conferred by the presence of the 5' UTR of HIV-1. The quantitative differences in the magnitude of translational inhibition by the 5' UTR in the different translation systems will be addressed in the Discussion.

mRNA stability

To examine the possibility that the observed differential translation efficiencies might be due to differential mRNA stabilities, ³H- or ³²P-labeled RNAs were incubated in reticulocyte lysate under the same conditions used for translation, or microinjected into *Xenopus* oocytes. The RNA was extracted at different time points and fractionated on formaldehyde agarose gels. The results are shown in Figure 3. In the reticulocyte lysate, the TARCAT, TAR(+111)-CAT, and TAR(+111R)CAT mRNAs are slightly less stable than the CAT mRNA (Figure 3A); all of the mRNAs are very stable in *Xenopus* oocytes (Figure 3B). Although small differences in mRNA stabilities are observed in reticulocyte

lysate, they cannot account for the differences in the amount of CAT protein synthesized. It is possible that the slight relative instability of the TARCAT mRNAs is attributable to poor translation and consequent lack of protection from nucleases by ribosomes (Losson and Lacroute *et al.*, 1979; Zitomer *et al.*, 1979).

Mutational analysis of the 5' UTR

To determine whether the inhibition of translation is a direct result of the predicted stem-loop structure shown in Figure 1B, we used site-directed mutagenesis to change nucleotides predicted to be base-paired. These changes should increase the translational efficiencies of the resulting mRNAs compared to TARCAT. Mutations on the opposite side of the stem designed to restore base pairing should restore inhibition of translation. The mutations chosen are shown in Figure 4, and the translation of the corresponding mRNAs in reticulocyte lysate in Figure 5. All the mutations designed to disrupt base pairing (TAR2A, TAR2C, TAR3, TAR2A3, TAR2R and TAR3R) lead to increases in translational efficiency of the corresponding mRNAs. The largest effect was seen with the combination of mutations TAR2A and TAR3 and TAR2A3, which leads to a 190-fold increase in CAT activity compared to TARCAT; TAR2A3 translated ~50% as efficiently as CAT in this experiment (compare lanes 2, 3 and 6 in Figure 5 and Table II). Both sets of compensatory mutations (TAR2R2C and TAR3R3) restore full inhibition of CAT translation (lanes 10 and 11). These results, as well as those from microinjected *Xenopus* oocytes, are summarized in Table II.

Cap structure accessibility of TARCAT mRNA is reduced

Another feature of the hairpin structure shown in Figure 1B, which could contribute to inhibition of translation, is inaccessibility of the cap structure. The cap structure must interact with eukaryotic initiation factors (eIFs) in order to allow for efficient binding of 40S ribosomal subunits (Edery *et al.*, 1987). To assess the efficiency of interaction of the cap structure of TARCAT mRNA with some of these initiation factors, such as eIF-4B, we used the UV-induced cross-linking assay (Pelletier and Sonenberg, 1985b). mRNA labeled with ³²P in the cap structure was incubated with reticulocyte lysate, followed by irradiation with UV light and RNase digestion. Labeled proteins were analyzed by SDS-PAGE and autoradiography and cap specificity was assessed by carrying out parallel incubations in the presence of the cap analogue, m⁷GDP. Results from such an assay using CAT, TARCAT, TAR2C and TAR2R2C mRNAs in reticulocyte lysate are shown in Figure 6. The intensity of crosslinking of eIF-4B (80 kd: indicated by an arrowhead)

Fig. 3. Analysis of mRNA stability. Translation reactions were performed as in Figure 2, and aliquots were withdrawn at the times indicated [in minutes for (A), in hours for (B)] at the bottom of the figure; after digestion with proteinase K (0.4 mg/ml) for 30 min at 37°C and ethanol precipitation, the RNA was analyzed by formaldehyde–agarose gel electrophoresis and fluorography (A) or autoradiography (B). A, reticulocyte lysate ([³H]RNA); B, *Xenopus* oocytes ([³²P]RNA). Lanes 1–3, CAT mRNA; 4–6, TARCAT mRNA; 7–9, TAR(+111)CAT mRNA; 10–12, TAR(+111R)CAT mRNA.

Fig. 4. Mutations in the HIV-1 TAR region. Boxes indicate areas in the stem–loop structure which were chosen for mutational analysis.

to the cap structure of TARCAT mRNA is lower (15-fold) than for CAT mRNA (compare lane 3 to 1). Mutation of nucleotides +2 and +3 in TAR2C, which disrupts the secondary structure at the base of the stem (Figure 4), leads to a significant increase (5-fold) in the efficiency of cross-linking of eIF-4B (compare lane 5 to 3); restoration of base-pairing in this region, in TAR2R2C, eliminates this effect (compare lane 7 to 5).

Discussion

The 5' +1 to +111 sequence of the HIV-1 viral genome and of HIV-1 mRNAs is shown to inhibit significantly the

Fig. 5. Translation of TARCAT mutant mRNAs in reticulocyte lysate. Conditions were as described in the legend to Figure 2A. SDS–PAGE analysis of ³⁵S-labeled products is shown. Lane 1, no mRNA; lane 2 CAT mRNA; lane 3, TARCAT; lane 4, TAR2A; lane 5, TAR3; lane 6, TAR2A3; lane 7, TAR2C; lane 8, TAR2R; lane 9, TAR3R; lane 10, TAR2R2C; lane 11, TAR3R3.

translation of a heterologous mRNA. The observation that mutations that are predicted to disrupt the secondary structure of this region relieve the translation inhibition, and that inhibition is restored when compensating mutations are introduced to reconstruct the predicted secondary structure,

Table II. Effect of mutations in the stem-loop structure formed by nucleotide +1 to +60 from the 5' UTR of HIV-1 on translational efficiency^a

Mutation ^b	ΔG° (kcal/mol) ^c	Relative translation efficiency	
		Reticuloctye lysate	<i>Xenopus</i> oocytes
None ^d	-36.0	1.0	1.0
TAR2A	-23.8	13	11
TAR2C	-23.8	9.0	3.2
TAR3	-31.8	17	5.1
TAR2A3	-19.6	190	11
TAR2R	-23.8	3.3	6.5
TAR3R	-31.8	57	9.0
TAR2R2C	-36.0	1.0	1.1
TAR3R3	-46.0	0.3	0.3

^aValues were determined by quantitation of acetylated and non-acetylated forms of chloramphenicol after CAT assays, and represent an average from two independent experiments.

^bSee Figure 4A.

^cEstimated using Tinoco's rules (Tinoco *et al.*, 1973); all values are $\pm 10\%$.

^dThe amount of CAT synthesized from TARCAT mRNA was set at 1.0.

Fig. 6. UV cross-linking of cap-labeled mRNAs to proteins in reticulocyte lysate. Cross-linking was carried out in the absence (–) or presence (+) of the cap analogue m^7GDP , and labeled proteins analyzed by SDS–PAGE and autoradiography. Lanes 1 and 2, CAT mRNA; lanes 3 and 4, TARCAT mRNA; lanes 5 and 6, TAR2C mRNA; lanes 7 and 8, TAR2R2C mRNA.

implies that at least part of the observed inhibition can be attributed to the secondary structure itself.

Inaccessibility of the 5' cap structure to cap binding proteins also appears to play a role in the inhibitory effect of HIV-1 5' UTR. The cap is not readily accessible to the eIF-4B protein as judged by UV cross-linking experiments (Figure 6). Mutations that disrupt the base pairing at the base of the stem-loop structure formed by the HIV-1 5' sequences increase the efficiency of both eIF-4B binding and translation. Compensatory mutations that restore base pairing decrease translation efficiency and eIF-4B binding. These results are consistent with the idea that secondary structure interferes with cap-mediated ribosome binding to the mRNA (Pelletier and Sonenberg, 1985a,b; Edery *et al.*, 1987).

Although the inhibitory effects of the 5' HIV-1 sequences

are evident in all three translation systems studied, several differences in the magnitude of the inhibitory effects and of the effects of mutations in the 5' HIV-1 LTR sequences are notable. Relief of translation inhibition by a combination of mutations at the base of the stem-loop structure and further up in the stem that disrupt base pairing (compare specifically the TAR2A, TAR3 and TAR2A3 mutants) have a multiplicative effect in reticulocyte lysate, but in *Xenopus* oocytes, TAR2A3 translates with the same efficiency as TAR2A. This apparent discrepancy may reflect a difference in the relative importance of two of the factors which determine translation efficiency: secondary structure and cap structure accessibility. The *Xenopus* oocyte appears to be more sensitive to changes in cap accessibility and less sensitive to secondary structure, compared to the reticulocyte lysate system. This hypothesis may also help to explain why mutations which disrupt secondary structure at the base of the stem-loop structure (e.g. TAR2A, TAR2R) have a greater effect on the relief of translation than do mutations further up the stem (e.g. TAR3) when examined in *Xenopus* oocytes, whereas a different situation is observed in reticulocyte lysate. An alternative explanation is that in the oocyte the CAT mRNAs have to compete with endogenous mRNA for translation factors, whereas in reticulocyte lysate the endogenous mRNA was digested with micrococcal nuclease. This might also help to explain the lower increase in translation efficiency in the oocyte as compared to reticulocyte lysate when a significant portion of the secondary structure in the TAR region was disrupted (e.g. TAR2A3). Competition between endogenous and exogenous mRNAs in the oocyte might lead to a situation where the amounts of translation factors other than those involved in mRNA binding to ribosomes become limiting.

One of the mutations (TAR3R3; Figure 4) exemplifies the correlation between secondary structure and translational efficiency. The mutant TAR3R3 mRNA is translated ~3-fold less efficiently than TARCAT mRNA. This difference is probably due to an increase in the stability of the secondary structure (by about –10 kcal/mol) due to the direct and co-operative effects on secondary structure of an additional GC pair (Figure 4A; Tinoco *et al.*, 1973). However, as discussed above for other mutations, other factors, such as cap accessibility, play a role in determining translational efficiency (Godefroy-Colburn *et al.*, 1985).

We also note that mutations which are predicted to have the same effect on disruption of secondary structure do not necessarily lead to a similar increase in relative translational efficiency (e.g. compare TAR2A with TAR2C, and TAR3 with TAR3R). This may be due to alternative conformations which the mutated RNAs may assume, depending on their exact sequence. In all cases, however, these conformations must be less stable than that of the wild-type UTR.

Our results confirm and extend the observation by Cullen (1986), using TAR–interleukin 2 hybrids transfected *in vivo*, that sequences from the HIV LTR 'R' region inhibit translation. In this latter study, sequences from +1 to +37 were found to be sufficient for inhibition. Although these sequences obviously cannot form the same structure shown in Figure 1B, they can form a small, but stable hairpin structure on their own (N.T.Parkin, unpublished observations).

The *tat* protein of HIV-1 does not relieve the observed translational block in *in vivo* extracts, or in microinjected *Xenopus* oocytes (unpublished observations). Assuming that the *tat* protein made in these systems is not lacking some kind of modification required for activity, this result suggests that the post-transcriptional component of *trans*-activation by *tat* (Cullen, 1986; Feinberg *et al.*, 1986; Rosen *et al.*, 1986; Wright *et al.*, 1986; Muesing *et al.*, 1987; Sadaie *et al.*, 1988) is either not effected at the translational level, or that it requires some other factor(s) or co-factor(s) for translational activity. However, it seems logical to predict that the block to translation of viral mRNAs by their 5' UTR must be overcome to allow for efficient viral structural protein synthesis and viral replication, and that this would make an important contribution to the magnitude of *trans*-activation by *tat* observed in experimental systems. Whether this effect could be mediated by *tat in vivo*, either directly or indirectly, remains to be determined. *Tat* was also reported to function as a transcriptional antiterminator (Kao *et al.*, 1987); the secondary structure of the TAR region may also be important for this effect.

The *cis*-acting inhibition of viral protein synthesis by the 5' sequences of HIV-1 mRNAs may play an important role in the life cycle of HIV. Infection by cells with HIV-1 is complex. Fully latent states of infection, states of infection in which low levels of virus are produced, and active lytic infections characterized by abundant production of virus, have all been observed (Curran *et al.*, 1985; Zagury *et al.*, 1986). Inhibition of the efficient utilization of viral mRNA due to the secondary structure of their 5' ends may be an important feature in establishment and maintenance of latent infections. Viewed in this context, *cis*-acting repression of viral mRNA translation might be a mechanism for avoidance of the host immune response, by control of the level of viral antigen production. A shift from latent to lytic infection is likely to involve a release of the *cis*-acting translational repression.

Materials and methods

Construction of plasmids

The SP6 constructs used to synthesize mRNAs are shown diagrammatically in Figure 1. The bacterial chloramphenicol acetyltransferase (CAT) coding sequence was subcloned from pSV2CAT (Gorman *et al.*, 1982) into the *Hind*III/*Bam*HI site of pSP64 (Melton *et al.*, 1984) to produce pSP64/CAT. To construct pSP64/TARCAT, a plasmid bearing HIV-1 nucleotides –17 to +80 fused 5' to the CAT coding sequence (Rosen *et al.*, 1985) was first

linearized at a unique *Cla*I site created at nucleotide –17. The linearized DNA was treated with *Bal*31 exonuclease, ligated to a *Pst*I linker and recircularized. The extent of each deletion was examined by DNA sequencing using the technique of Maxam and Gilbert (1980). One clone, shown to contain a deletion of nucleotides –17 to +1 was cleaved with *Pst*I and *Bam*HI, and the resultant +1 to +80/CAT fragment was cloned into the *Pst*I/*Bam*HI site of pSP64. This construct contains 23 nt between the SP6 promoter and the +1 nucleotide of the TAR element (mostly polylinker sequences). To remove those intervening sequences, the *Sph*I–*Sac*I fragment, which contains the SP6 promoter and the first 39 nt of the TAR element, was subcloned into M13mp19. Oligonucleotide-directed deletion mutagenesis was performed (Zoller and Smith, 1982) using a 37 mer oligonucleotide complementary to sequences on either side of the deletion. The deletion was confirmed by sequencing (Sanger *et al.*, 1977) and the *Sph*I–*Sac*I fragment was subcloned back into pSP64. Transcription by SP6 RNA polymerase of this construct generates mRNA which begins at the +1 nucleotide of the HIV-1 LTR and thus has the same nucleotide sequence at its 5' end as HIV-1 mRNAs synthesized *in vivo*. A synthetic DNA fragment

5'-AGCTTGCCCTTGAGTGCTTCAAGTAGTGTGTGCCCTCGAGA-3'
3'-ACGGAACACACGAAGTTCATCACACGGGAGCTCTTCGA-5'

containing HIV-1 leader sequences from +80 (*Hind*III) to +111, followed by artificial *Xho*I and *Hind*III sites, was inserted at the *Hind*III site of pSP64/TARCAT to generate pSP64/TAR(+111)CAT. pSP64/TAR(+111)CAT contains the synthetic DNA fragment in the opposite orientation. Site-directed mutations in the TAR region were made using the two-primer method in M13 (Zoller and Smith, 1982); all mutations were confirmed by sequencing using the dideoxy chain termination method (Sanger *et al.*, 1977; Zhang *et al.*, 1988), before and after subcloning in pSP64.

Transcription and translation

SP6 plasmids were linearized with *Bam*HI and used as templates for transcription by SP6 RNA polymerase as described (Pelletier and Sonenberg, 1985a), except that the concentrations of GTP and the cap analogue m⁷GpppG were raised to 0.2 and 1.0 mM respectively. mRNA was labeled with [5-³H]CTP, and purified as described (Pelletier and Sonenberg, 1985a). The sp. act. of the mRNA was calculated to be 1.6×10^5 c.p.m./μg. RNA was routinely examined on formaldehyde–agarose gels, and found to consist of only one species of the expected size (~1.6 kb). *In vitro* translation of equimolar amounts of the various CAT mRNAs (i.e. equal amounts of radioactivity) was performed in reticulocyte lysate (Pelham and Jackson, 1976) or HeLa cell extract (Rose *et al.*, 1978). The concentration of mRNA used was 2.5 μg/ml, which was determined to be sub-saturating. Translation products were labeled with [³⁵S]methionine and analyzed by 15% SDS–PAGE. The amount of CAT product translated was determined by assaying for its enzymatic activity (Gorman *et al.*, 1982).

Microinjection in *Xenopus* oocytes

Stage 6 oocytes were isolated from mature *Xenopus* females, collagenase treated and maintained in modified Barth's saline as described (Colman, 1984b). After microinjection with mRNA (10 ng), oocytes were incubated at 21°C for up to 12 h, then homogenized in 0.25 M Tris–HCl, pH 7.6. The homogenates were centrifuged for 5 min at 12000 *g* at 4°C, and the supernatants assayed for CAT activity (Gorman *et al.*, 1982). Between 5 and 10 oocytes were injected with each mRNA species; to control for variations in the microinjection technique, the extracts were counted and equal counts, representing approximately one oocyte, were used in the CAT assay. However, for oocytes injected with CAT mRNA, the equivalent of one-tenth of an oocyte was used to keep the assay in the linear range. RNA stability was assessed by injecting and then extracting [³²P]mRNA as described (Colman, 1984a), at various times after injection and analysis on formaldehyde–agarose gels.

UV cross-linking

Crosslinking experiments were carried out essentially as described (Pelletier and Sonenberg, 1985b) with 2 μl of reticulocyte lysate and 30000 c.p.m. of cap-labeled mRNA (3×10^6 c.p.m./μg), and analyzed on a 12.5% SDS–polyacrylamide gel; labeled proteins were visualized by autoradiography.

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