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In vitro deadenylation of mammalian mRNA by a HeLa cell 3′ exonuclease

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We have identified a 3′ exonuclease in HeLa cell extracts which deadenylates mammalian mRNA and leaves the mRNA body intact after poly(A) removal. Only homopolymeric adenosine tails located at the 3′ end were efficiently removed by the exonuclease. The poly(A) removing activity did not require any specific sequences in the mRNA body either for poly(A) removal or for accumulation of the deadenylated mRNA. We conclude that the poly(A) removing activity is a 3′ exonuclease since (i) reaction intermediates gradually lose the poly(A) tail, (ii) degradation is prevented by the presence of a cordycepin residue at the 3′ end and (iii) RNAs having internally located poly(A) stretches are poor substrates for degradation. The possible involvement of the poly(A) removing enzyme in regulating mRNA translation and stability is discussed.

Key words: poly(A) removal/mRNA degradation/RNA processing

Introduction

The function of the poly(A) tail of eukaryotic mRNA has remained unknown since its discovery (Kates, 1970; Lim and Canellakis, 1970). It has been suggested that the poly(A) tail is involved in mRNA processing, transport, stability and translation (Brawermann, 1981; Bernstein and Ross, 1989; Jackson and Standart, 1990). Recently, several studies have highlighted the importance of the poly(A) tail during mRNA translation and mRNA decay. In developing eggs of *Xenopus laevis* it has been found that polyadenylated mRNAs are associated with ribosomes (Hyman and Wormington, 1988; McGrew *et al.*, 1989). Based on this finding and the identification of mRNAs gaining and losing poly(A) tails (Fox *et al.*, 1989; Fox and Wickens, 1990; Varnum and Wormington, 1990; Wickens, 1990b) it has been postulated that translation in developing eggs of *X.laevis* is regulated by varying the length of the poly(A) tail. The removal of the poly(A) tail suggests that a poly(A) removing activity is present. Indeed, evidence for such an activity has been provided by microinjecting adenylated RNA into the cytoplasm of *X.laevis* oocytes and showing that poly(A) tails are removed (Fox and Wickens, 1990; Varnum and Wormington, 1990). Similarly, it has been suggested that the poly(A) tails of several eukaryotic mRNAs are required for translation and proper gene regulation (Muschel *et al.*, 1986; Huarte *et al.*, 1987; Paek and Axel, 1987; Robinson *et al.*, 1988; Sachs and Davis, 1989; Vassalli *et al.*, 1989; Munroe and Jacobson, 1990). The function of the poly(A) tail during mRNA decay in mammalian cells has been extensively studied (reviewed in Bernstein and Ross, 1989). It has been shown that removal of the poly(A) tail precedes mRNA degradation and it has been proposed that 3′ exonuclease activities are required for poly(A) tail removal (Brewer and Ross, 1988; Wilson and Treisman, 1988; Swartwout and Kinniburgh, 1989; Shyu *et al.*, 1991). Thus, in many different types of cells, gene expression appears to some extent to be post-transcriptionally regulated by poly(A) tail removal. Furthermore, this suggests that a poly(A) tail removing enzyme should be present in most cell types.

Here we demonstrate the existence of a HeLa cell 3′ exonuclease activity. We have partly purified the activity and investigated its substrate requirements *in vitro*. Only homopolymeric adenosine tails located at the 3′ end of an mRNA are efficiently removed by the enzyme and the mRNA body is left intact after poly(A) removal. We conclude that the characterized activity has the properties of a mammalian poly(A) tail removing enzyme.

Results

RNA substrates

To identify and characterize a nuclease that degrades poly(A) tails of mammalian mRNAs, we synthesized *in vitro* (see Materials and methods) RNA substrates having 5′ capped RNA bodies followed by 30 residues of adenosine [L3(A_{30}), L3G(A_{30}) ML65(A_{30})], guanosine [ML43(G_{30})], uridine [ML54(U_{30})] or 32 cytidine residues [ML40(C_{32})] (Figure 1). Thus, each RNA substrate contained bona fide homopolymeric tails at their 3′ ends. The RNA bodies of L3(A_{30}) and L3G(A_{30}) were 54 nucleotides long and included sequences preceding the L3 polyadenylation site of human adenovirus type 2. The two RNAs were identical except for the AAUAAA sequence element which was

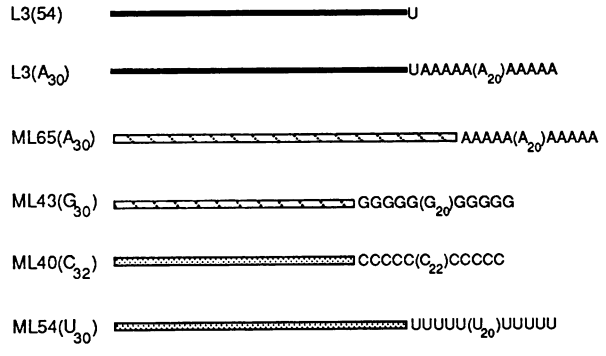

Fig. 1. Schematic drawing of RNA substrates. The relative sizes of RNA bodies and homopolymeric tails are indicated. The black bar corresponds to sequences preceding the adenovirus L3 polyadenylation site. Hatched and dotted bars correspond to the + and − strands of the multilinker sequences of plasmid pBS(−), respectively.

mutated to AAGAAA in the L3G(A_{30}) RNA. The AAUAAA sequence element is required for polyadenylation (reviewed in Wickens, 1990a). RNA bodies denoted by ML were obtained from the multilinker of plasmid vector pBS(−) (Stratagene) and were 65, 54, 43 or 40 nucleotides long, as indicated.

Deadenylation in vitro

We detected low poly(A) removing activity when the L3(A_{30}) RNA substrate was incubated in nuclear extract (NE) in conditions for *in vitro* polyadenylation. The conditions for *in vitro* deadenylation were optimized (see Materials and methods) and we found that Mg^{2+} and monovalent ions (K^+ or Na^+) were required for efficient deadenylation at pH 8.2. No deadenylation was detected when Mg^{2+} was replaced by Mn^{2+} (data not shown). The L3(A_{30}) RNA substrate was incubated in nuclear extract (NE) or cytoplasmic extract (S100) in conditions for *in vitro* deadenylation. Deadenylation and accumulation of an RNA fragment corresponding in size to the RNA body occurred when NE was used (Figure 2). To purify the nuclease further we fractionated NE by DEAE−Sephacel anion chromatography (see Materials and methods). Three fractions were collected and named I, II and III, respectively. The deadenylation activity was recovered in fraction II (Figure 2). This fraction was used as the source for deadenylation activity in the experiments described below.

Deadenylation requires no specific sequence elements besides the poly(A) tail

The involvement of the RNA body in deadenylation was tested by comparing deadenylation of the L3(A_{30}) and ML65(A_{30}) RNA substrates. Both RNA substrates were deadenylated with similar efficiencies (Figure 3A and B). Thus, a specific sequence element in the RNA body did not seem to be required for either deadenylation or accumulation of the deadenylated RNA body. We also tested the involvement of the polyadenylation signal AAUAAA by incubating the L3G(A_{30}) RNA substrate in conditions for deadenylation and found that this RNA substrate was efficiently deadenylated (data not shown).

To investigate further the specificity of the deadenylating activity, we incubated the RNA substrates ML43(G_{30}), ML40(C_{32}) and ML54(U_{30}) in conditions for deadenylation. No degradation of tails consisting of guanosine or uridine residues was seen (Figure 3C and D). Homopolymeric tails consisting of cytosine residues were removed with a very low efficiency compared with adenosine tails (Figure 3E). Thus, the activity showed strong preference for degrading homopolymeric adenosine tails.

The deadenylation activity degrades the poly(A) tail and leaves the mRNA body intact

To investigate the reaction pathway, we incubated L3(A_{30}) RNA (labeled by inclusion of [α-^{32}P]ATP during *in vitro* transcription) for 20 min in conditions for deadenylation. The reacted RNA was fractionated by gel electrophoresis and RNA representing different stages of deadenylation was excised and purified. The purified reaction intermediates

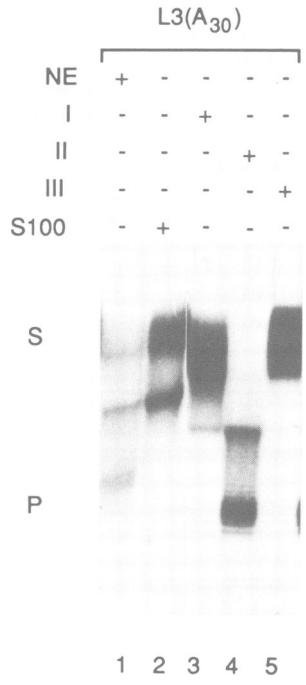

Fig. 2. Identification of an exonuclease activity in HeLa cell extract. Nuclear extract (NE), fractions of nuclear extract (I, II and III) or cytoplasmic extract (S100) were incubated together with L3(A_{30}) RNA substrate in conditions for deadenylation. Reacted RNA was purified and fractionated by gel electrophoresis. + indicates the presence of NE, fractions I, II and III or S100. S and P denote the locations of substrate and product RNAs, respectively.

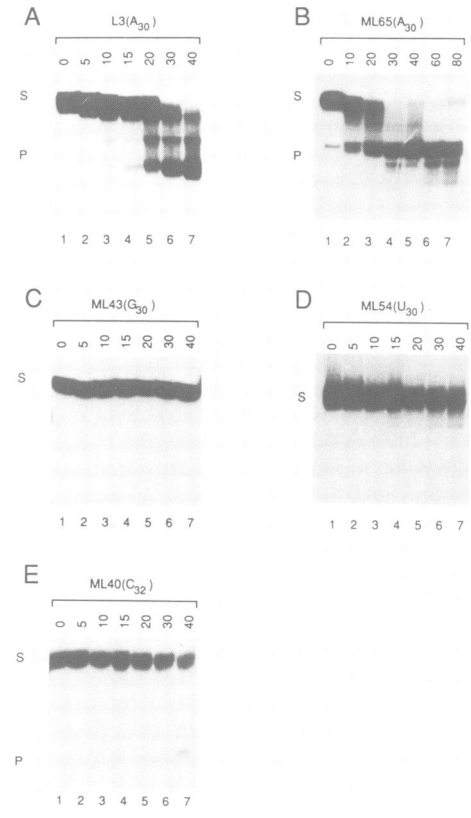

Fig. 3. Substrate requirement for degradation by the 3′ exonuclease. RNA substrates were incubated in conditions for deadenylation. Reactions were terminated at the indicated time points. As RNA substrates, L3(A_{30}) (**A**), ML65(A_{30}) (**B**), ML43(G_{30}) (**C**), ML54(U_{30}) (**D**) and ML40(C_{32}) (**E**) were used. S and P denote the locations of substrate and product RNAs, respectively. Numbers above lanes indicate length of incubation in minutes.

were digested with T1 nuclease and subsequently separated by gel electrophoresis. To obtain fragments representing intact or completely deadenylated RNAs, L3(A_{30}) and L3(54) were treated with T1 nuclease. The T1 digestion pattern of L3(54) has been described by Moore *et al.* (1986). The poly(A) tail-containing T1 fragment consists of the two 3′ located nucleotides of the mRNA body followed by the poly(A) tail. Figure 4 (panel A) shows that the intermediates of the deadenylation reaction corresponded to RNA molecules that were shortened at their 3′ ends and that the resulting RNA product corresponded to the RNA body. The latter conclusion was based on the finding that the T1 patterns of the deadenylated product and L3(54) RNA were identical.

Efficient deadenylation requires 3′ located poly(A) tails and the presence of a 3′ hydroxyl

To characterize further the substrate requirement during deadenylation, we performed two sets of experiments. First, RNA substrates having 15 [L3(A_{30})X_{15}], 49 [L3(A_{30})X_{49}] or 164 [L3(A_{30})X_{164}] nucleotides following the poly(A) tail of L3(A_{30}) RNA were incubated in deadenylating conditions. No deadenylation was seen when the L3(A_{30})X_{164} RNA substrate was used (Figure 4B). However, some shortening of the RNA substrate was detected since a slight reduction in the length of the RNA was observed. When the L3(A_{30})X_{15} and L3(A_{30})X_{49} RNA substrates were used, some accumulation of RNA corresponding to the L3 RNA body occurred (Figure 4B). Thus, although some activity was detected when the poly(A) tract was followed by a short stretch of nucleotides, efficient deadenylation was only observed when the poly(A) tail was located at the 3′ end of the RNA substrate.

Secondly, L3(A_{30}) RNA substrate was incubated in two parallel sets of reactions in deadenylating conditions for 0, 10, 20, 40, 50, 60, 80 and 120 min. After 40 min of incubation, either L3(A_{30}) or L3(A_{30}) ending with one ^{32}P-labeled cordycepin residue (3′ dATP) at the 3′ end was added. Figure 4C shows that the cordycepin labeled RNA substrate was not deadenylated (lanes 14–18) although deadenylating activity was present before the cordycepin labeled RNA was added (lanes 11–13) and still present as shown in the parallel reaction (lanes 6–10). Thus, deadenylation was prevented by the absence of a 3′ hydroxyl.

Discussion

In this paper we have identified a 3′ exonuclease in HeLa cells which has properties of a mammalian mRNA poly(A) tail removing enzyme. We base this conclusion on the observations that only homopolymeric adenosine tails located at the 3′ end were efficiently degraded and that the mRNA body was left intact after the poly(A) tail was removed (Figure 3A and B; Figure 4A). We also observed that the poly(A) removing activity was independent of specific sequences in the mRNA body for either deadenylation or accumulation of the deadenylated mRNA (Figure 3). We conclude that the poly(A) removing enzyme is a 3′ exonuclease since (i) reaction intermediates gradually lose the poly(A) tail (Figure 3A and B; Figure 4A), (ii) RNAs having internally located poly(A) stretches are poor substrates for deadenylation (Figure 4B) and (iii) poly(A) tail removal can be prevented by the presence of a cordycepin residue at the 3′ end (Figure 4C). These observations do not show

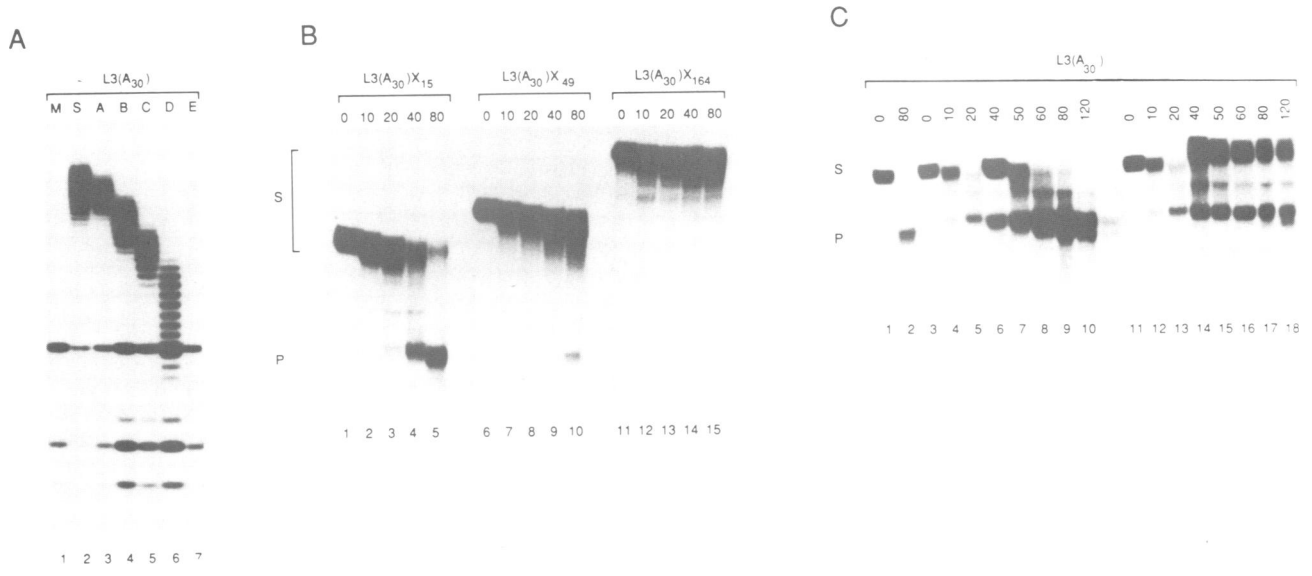

Fig. 4. Evidence for 3′ exonuclease activity. (**A**) T1 nuclease digestion of reaction intermediates obtained from deadenylation of L3(A_{30}) RNA substrate, labeled using [α-^{32}P]ATP during *in vitro* transcription. Reaction intermediates present after 20 min of incubation in conditions for deadenylation were purified, named A to E (where A represents the 'longest' and E the 'shortest' intermediate) and digested with T1 nuclease. T1 nuclease resistant RNA fragments of L3(54) (lane 1), L3(A_{30}) (lane 2) and reaction intermediates (lanes 3–7) were separated by gel electrophoresis. (**B**) Deadenylation of RNA substrates containing internally localized poly(A) stretches. RNA substrates L3(A_{30})X_{15} (lanes 1–5), L3(A_{30})X_{49} (lanes 6–10) and L3(A_{30})X_{164} (lanes 11–15) were incubated in deadenylating conditions for the indicated time. S and P denote the locations of substrate and product RNAs, respectively. Numbers above lanes indicate length of incubation in minutes. (**C**) Deadenylation of RNA L3(A_{30}) having a cordycepin residue at its 3′ end. Lanes 3–18 show deadenylation in reactions containing L3(A_{30}) RNA substrate. After 40 min of incubation the reactions were supplemented by addition of L3(A_{30}) RNA substrate (lanes 6–10) or cordycepin modified L3(A_{30}) RNA (lanes 14–18). In lanes 1 and 2 deadenylation of radioactively labeled L3(A_{30}) was competed by addition of nonlabeled L3(A_{30}) RNA in molar amounts corresponding to the amount of RNA added to reactions separated in lanes 14–18. S and P denote the locations of substrate and product RNAs, respectively. Numbers above lanes indicate length of incubation in minutes.

unambiguously that the poly(A) removing activity is a 3′ exonuclease that removes one adenosine residue at a time. However, we have recently observed that adenosine residues accumulate as the reaction proceeds, indicating that the activity liberates mononucleotides (J.Åström, A.Åström and A.Virtanen, unpublished observation). The small amount of degradation we detected using the ML40(C_{32}) RNA substrate or the RNA substrates having internally located poly(A) stretches was probably due to an unspecific nuclease present in our crude enzyme preparation. In the case of the RNA substrates having internally located poly(A) stretches, the time course experiments suggested that the poly(A) tails were rapidly removed when the nucleotides following the poly(A) stretches were degraded (Figure 4B).

The highest deadenylating activity was found in a fraction obtained by anion exchange chromatography of NE. However, it is not yet possible to predict the subcellular localization of the 3′ exonuclease from these experiments. For instance, the poly(A) removing enzyme may be associated with cytoplasmic components that are sedimented during the initial centrifugation steps of NE preparation.

To our knowledge this is the first time an activity with properties of a mammalian poly(A) tail removing enzyme has been identified and characterized *in vitro*. Mammalian nucleases degrading poly(A) *in vitro* have previously been described by Lazarus and Sporn (1967) and by Abraham and Jacob (1978). Moreover, Brewer and Ross (1988) have detected a poly(A) degrading activity in an *in vitro* mRNA decay system. However, in none of these studies has it been demonstrated that the poly(A) degrading activities exclusively degrade the poly(A) tail of an mRNA and leave the mRNA body intact after poly(A) tail removal.

Deadenylation of mRNA has been observed to occur in the cytoplasm of frog and mammalian cells and it has been suggested that deadenylation may control translation as well as mRNA stability (Bernstein and Ross, 1989; Wickens, 1990b). In developing frog and mouse cells, polyadenylation/deadenylation is most likely involved in regulating stage specific translation. It has recently been shown that the deadenylation reaction in frog cells does not require the presence of a unique sequence in the RNA besides the poly(A) tail (Fox and Wickens, 1990; Varnum and Wormington, 1990). In the light of this finding, the poly(A) removing enzyme we have detected is likely to be involved in a similar polyadenylation/deadenylation process which could indirectly regulate translation in human somatic cells. It has been shown that mRNA decay in mammalian cells requires several independent steps to occur and that poly(A) removal precedes cytoplasmic mRNA degradation. Furthermore, it has been suggested that the rate of poly(A) removal is a major factor determining the half-life of mRNA (Mercer and Wake, 1985; Brewer and Ross, 1988; Wilson and Treisman, 1988; Bernstein *et al.*, 1989; Bernstein and Ross, 1989; Swartwout and Kinniburgh, 1989; Shyu *et al.*, 1991). The activity we have characterized may be the poly(A) tail removing enzyme active during degradation of mRNA. Thus, the poly(A) removing enzyme may be involved in regulating the half-life of an mRNA.

Detailed *in vitro* mechanistic studies of poly(A) removal in mammalian cells, using the HeLa cell 3′ exonuclease described here, are now at hand. The characterization and purification of the 3′ exonuclease will make it possible to study the functional importance of poly(A) removal *in vivo*.

Materials and methods

Cells and preparation of cell extract

HeLa cells were grown in Dulbecco's minimal essential medium supplemented with 10% newborn calf serum and harvested in exponential phase (5×10^5 cells/ml). Nuclear extract and the S100 fraction were prepared essentially according to Dignam *et al.* (1983) with the modifications of Moore and Sharp (1985). The nuclei were extracted with 1 ml of buffer C (20 mM HEPES−KOH, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol; pH 8.2) per ml of collected cell pellet. The nuclear extract and the S100 fraction were dialysed against buffer D (20 mM HEPES−KOH, 100 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol; pH 8.2) in dialysis tubing with molecular weight cut-off at 6−8000 (Spectra/Por 1 no. 132650) for 5 h. After dialysis the nuclear extract was frozen in liquid nitrogen and stored at −70°C. The nuclear extract and the S100 fraction contained ~13 and 21 mg protein/ml, respectively. Protein concentration was determined using the Bio-Rad protein assay kit (no. 500-0001) and bovine gamma globulin as reference.

Fractionation by DEAE−Sephacel chromatography

A DEAE−Sephacel ion exchange column (Pharmacia no 17−0500) with a bed volume of 100 ml and a diameter of 5 cm was equilibrated with buffer D (see above) at a flow rate of 9 cm/h. Approximately 325 mg (25 ml) of nuclear extract was thawed, centrifuged at 20 000 *g* for 10 min and applied to the column at a flow rate of 9 cm/h. Two successive step elutions were performed with buffer D containing 0.15 M and 1.0 M KCl. The column was washed with 250 ml of the current elution buffer before the next step elution was performed. Aliquots of the eluate were assayed for protein. Protein that eluted in the flowthrough and each step fraction was combined as fractions I (2.9 mg/ml), II (1.2 mg/ml) and III (4.2 mg/ml). Fraction I was frozen directly and fractions II and III were dialysed against buffer D for 5 h. Fractions were frozen in liquid nitrogen and stored at −70°C.

Plasmids

Plasmids pT3L3(A_{30}) and pT3L3G(A_{30}) were constructed by annealing the complementary oligonucleotides AV13 (5′-AGCTT GTAGG AGACA C-TTTC AATAA AGGCA AATGT TTTTA TTTGT AAAAA AAAAA AAAAA AAAAA AAAAA AAAAA TGCAT CTGCA-3′), and AV14 (5′-GATGC ATTTT TTTTT TTTTT TTTTT TTTTT TTTTT TACAA ATAAA AACAT TTGCC TTTCT TGAAA GTGTC TCCTA CA-3′). The annealed oligonucleotides were inserted between the *Hin*dIII and the *Pst*I restriction sites of the pBS(−) plasmid vector (Stratagene). Ligated plasmids were transfected into *E.coli* HB101. Clones containing recombinant plasmids were identified, plasmid DNA was purified and subjected to a second transfection step. Pure clones of the two different oligonucleotides were isolated (Sambrook *et al.*, 1989). The isolated plasmids which contained oligonucleotides AV13 and AV14 were named pBSL3(A_{30}) and pBSL3G(A_{30}), respectively.

Plasmid pT3ML65(A_{30}) was constructed by inserting annealed oligonucleotides AA13 (5′-AATTC AAAAA AAAAA AAAAA AAAAA AAAAA AAAAA TGCATG-3′) and AA14 (5′-AATTC ATGCA TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT G-3′) into the *Eco*RI site of plasmid pBS(−) (Stratagene). Plasmid DNA having the *Nsi*I site of the insert distal to the T3 RNA polymerase promoter of the vector was isolated.

Plasmid pT3ML43(G_{30}) was constructed by inserting annealed oligonucleotides JA1 (5′-CTAGA CTGCA GGGGG GGGGG GGGGG GGGGG GGGGG GGGGG GGCCC T-3′) and JA2 (5′-CTAGA GGGCC CCCCC CCCCC CCCCC CCCCC CCCCC CCCCC TGCAG T-3′) into the *Xba*I site of plasmid pBS(−). Plasmid DNA having the *Apa*I site of the insert distal to the T3 RNA polymerase promoter of the vector was isolated. The identity of each clone was verified by DNA sequence analysis (Maxam and Gilbert, 1977; Sanger *et al.*, 1977).

ML54(U_{30}) DNA was obtained by PCR amplification (Saiki *et al.*, 1988) of pT3L3(A_{30}) using oligonucleotides JA4 (5′-AAAAA AAAAA AAAAA AAAAA AAAAA AAAAA TGCAT-3′) and JA5 (5′-AGTGA ATTGT AATAC GACTC ACTAT AG-3′) as primers. The obtained DNA product was directly used for *in vitro* transcription.

In vitro transcription

5′ end capped (^{7}mG(5′)ppp(5′)G, Pharmacia no. 27-4635) (Moore and Sharp, 1985) RNA substrates were synthesized by *in vitro* transcription using T3 or T7 RNA polymerases (Stratagene) according to the manufacturer's instructions in the presence of DNA template and [α-^{32}P]UTP [DuPont/NEN NEG-007X, 40 Ci/mmol in the transcription mixture, 5 Ci/mmol for ML54(U_{30})] or when indicated [α-^{32}P]ATP (DuPont/NEN NEG-003H, 2.5 Ci/mmol in the transcription mixture). Transcribed RNA was purified according to Moore and Sharp (1985). L3(54), L3(A_{30}),

L3G(A_{30}), ML65(A_{30}) and ML43(G_{30}) RNAs were obtained by *in vitro* transcription using T3 RNA polymerase and plasmid pT3L3 (Skolnik-David *et al.*, 1987) digested with *Rsa*I, plasmids pT3L3(A_{30}), pT3L3G(A_{30}) and pT3ML65(A_{30}) digested with *Nsi*I and plasmid pT3ML43(G_{30}) digested with *Apa*I. ML40(C_{32}) and ML54(U_{30}) RNAs were obtained by *in vitro* transcription using T7 RNA polymerase and plasmid pT3ML43(G_{30}) digested with *Pst*I or ML54(U_{30}) DNA. L3(A_{30})X_{15}, L3(A_{30})X_{49} and L3(A_{30})X_{164} RNA substrates were obtained by *in vitro* transcription using T3 RNA polymerase and plasmid pT3L3(A_{30}) digested with *Hin*cII, *Eco*RI and *Pvu*II, respectively.

In vitro deadenylation

Conditions for *in vitro* deadenylation were: 1 mM $MgCl_2$, 2.5% (w/v) polyvinyl alcohol (Sigma P-8136, mol. wt 10 000), 200 mM KCl, 0.15 units RNAguard (Pharmacia no. 27-0815), 5–20 fmol RNA substrate and 48% (v/v) buffer (20 mM HEPES–KOH, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol; pH 8.2). The reaction volume was 25 μl and incubations were performed at 30°C. The amounts of protein used were 26 μg (NE), 5.8 μg (fraction I), 2.4 μg (fraction II), 8.4 μg (fraction III) and 42 μg (S100) respectively. Reactions were terminated and RNA was purified according to Moore and Sharp (1985). RNA was analyzed by electrophoresis in 10% polyacrylamide (19:1 acrylamide/bisacrylamide)–7 M urea gels followed by autoradiography of the resulting gel.

T1 nuclease digestion

Reaction intermediates were purified by eluting RNA from a 10% polyacrylamide (19:1 acrylamide/bisacrylamide)–7 M urea gel (Moore and Sharp, 1985). T1 nuclease digestion of RNA was performed in 25 μl of: 25 mM Tris–HCl (pH 7.8), 50 mM NaCl, 10 mM $MgCl_2$, 100 mg/ml BSA, 2 mM β-mercaptoethanol and 160 units/ml T1 (Calbiochem no. 556785) for 30 min at 37°C. Treated RNA was purified by phenol:chloroform extraction and precipitated by ethanol.

Addition of cordycepin

Addition of cordycepin to L3(A_{30}) RNA substrate was done as follows: non-radioactive L3(A_{30}) RNA was labeled by addition of ^{32}P-labeled cordycepin (3′-dATP, DuPont/NEN, NEG 026) to the 3′ end of the RNA using *E.coli* poly(A) polymerase (Pharmacia no. 27-0206) according to the manufacturer's instructions.

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