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Regulated poly(A) tail shortening in somatic cells mediated by cap-proximal translational repressor proteins and ribosome association

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ABSTRACT

The poly(A) tail plays an important role in translation initiation. We report the identification of a mechanism that operates in mammalian somatic cells, and couples mRNA poly(A) tail length with its translation state. The regulation of human ferritin L-chain mRNA by iron-responsive elements (IREs) and iron regulatory proteins (IRPs) is subject to this mechanism: translational repression imposed by IRP binding to the IRE of ferritin L-chain mRNA induces poly(A) tail shortening. For the accumulation of mRNAs with short poly(A) tails, IRP binding to an IRE per se is not sufficient, but must cause translational repression. Interestingly, puromycin and verrucarin (general translation inhibitors that dissociate mRNAs from ribosomes) mimick the negative effect of the specific translational repressor proteins on poly(A) tail length, whereas cycloheximide and anisomycin (general translation inhibitors that maintain the association between mRNAs and ribosomes) preserve long poly(A) tails. Thus, the ribosome association of the mRNA appears to represent the critical determinant. These findings identify a novel mechanism of regulated polyadenylation as a consequence of translational control. They reveal differences in poly(A) tail metabolism between polysomal and mRNP-associated mRNAs. A possible role of this mechanism in the maintenance of translational repression is discussed.

Keywords: iron-responsive element; mRNA; poly(A) tail; RNA processing; RNA/protein interaction; translational control

INTRODUCTION

Modifications of the 5′ and 3′ termini of eukaryotic mRNAs are required for efficient translation in the cytoplasm. Although the addition of the 5′ ^{7}mGpppN cap structure occurs in the nucleus, the poly(A) tail is appended in the nucleus and then subject to length alterations in the cytoplasm. A positive correlation between poly(A) tail length and translational efficiency has been established in cell-free translation extracts (Munroe & Jacobson, 1990; Iizuka et al., 1994; Tarun & Sachs, 1995), cultured mammalian, plant, and yeast cells (Gallie, 1991), as well as in oocytes from vertebrate and invertebrate species (Rosenthal et al., 1983; Vassalli et al., 1989; Wickens, 1992; Curtis et al., 1995).

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During oocyte maturation, fertilization, and early embryonic development, regulated polyadenylation serves as one of the main mechanisms for gene regulation (discussed in Jackson & Standart, 1990; Wickens, 1992; Wormington, 1993; Hentze, 1995) by inducing a switch of maternal mRNAs from a translationally dormant to a translationally active state. For example, polyadenylation of bicoid mRNA precedes bicoid protein expression and is required for the formation of anterior structures in *Drosophila* embryos (Sallés et al., 1994). Similarly, polyadenylation of c-mos mRNA controls the meiotic maturation of *Xenopus* and murine oocytes (Gebauer et al., 1994; Sheets et al., 1995). The sequences that control poly(A) addition [cytoplasmic polyadenylation elements, (CPEs)] are located in the 3′UTRs at various distances upstream of the poly(A) signal, and transcripts lacking CPEs undergo default poly(A) removal during *Xenopus* oocyte maturation (Fox & Wickens, 1990; Varnum & Wormington, 1990; Standart & Dale, 1993). Changes in poly(A) tail length have also been observed in somatic cells in response to

extracellular stimuli of growth and differentiation or viral infection (i.e., Muschel et al., 1986; Paek & Axel, 1987; Carrazana et al., 1988; Robinson et al., 1988; Zingg et al., 1988; Carter & Murphy, 1989; Murphy & Carter, 1992; Dehlin et al., 1996), although little is known about the underlying mechanism(s). Following the transport of the mRNA to the cytoplasm, poly(A) tails of stable mRNAs are gradually shortened to 40–65 adenosines (Brawerman, 1981; Mercer & Wake, 1985; Shyu et al., 1991). The mean steady-state poly(A) tail length can vary considerably between different mRNA species (Morrison et al., 1979; Palatnik et al., 1979). Physiological stimuli that influence the rate of poly(A) tail shortening of stable transcripts have not been reported so far. In contrast, unstable mRNAs, like the c-fos mRNA, are deadenylated rapidly and degraded subsequently (Shyu et al., 1991; Beelman & Parker, 1995).

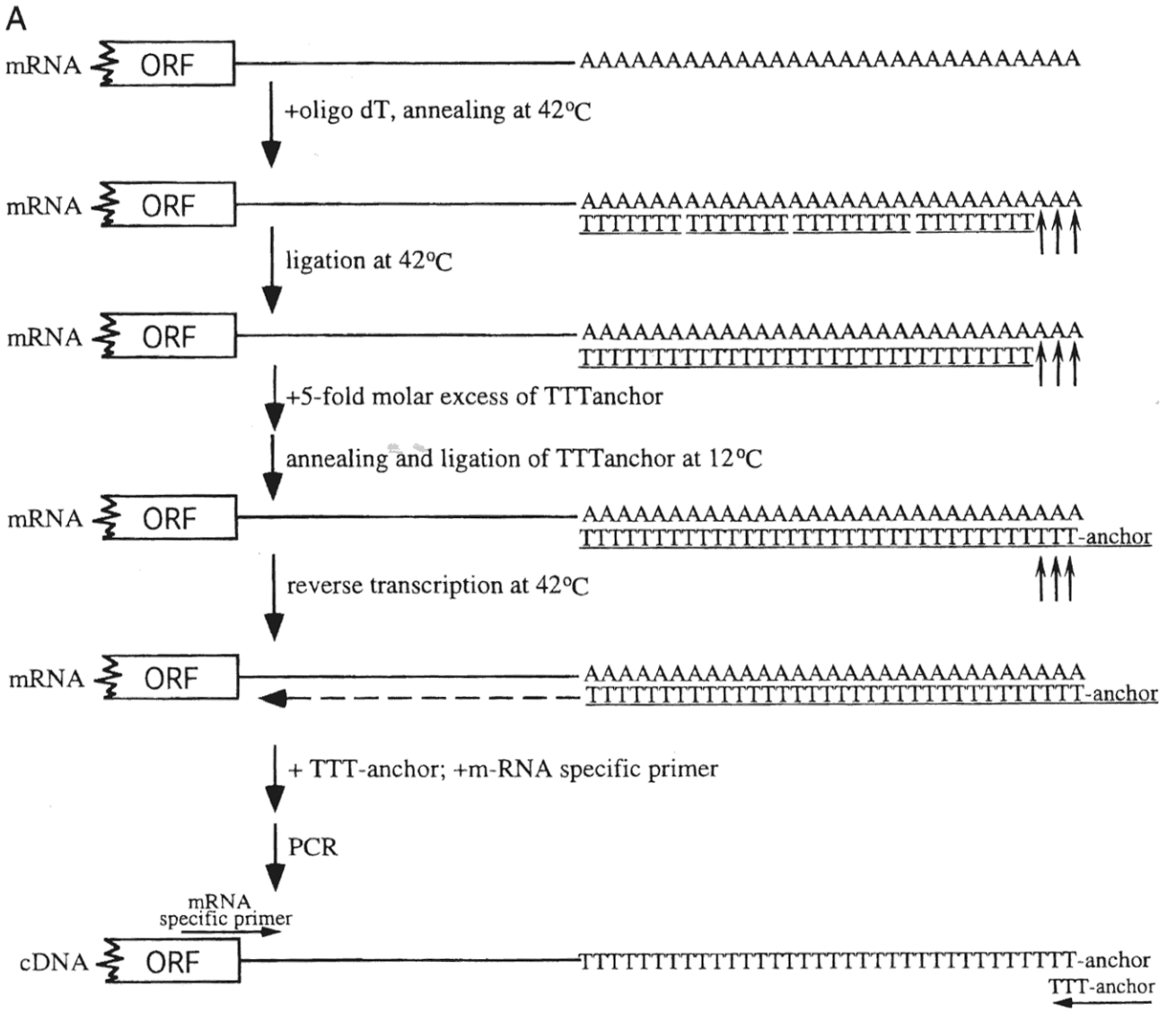
Translation can also be regulated at the 5′ end of mRNAs. The binding of iron regulatory proteins (IRP-1 and IRP-2) to iron-responsive mRNA elements (IREs) provides an intensively studied example of this control mechanism (Hentze & Kühn, 1996). Iron deficiency or exposure of cells to nitric oxide or H_2O_2 induce IRP binding to the IREs and translational repression of ferritin and other IRE-containing target mRNAs (Martins et al., 1995; Pantopoulos & Hentze, 1995a, 1995b). In cell-free translation extracts from rabbit reticulocytes and wheat germ, IRE/IRP-1 complexes inhibit the stable association of the 43S pre-initiation complex with the mRNA (Gray & Hentze, 1994). This inhibition appears to result from steric hindrance rather than a direct interaction between IRP-1 and a translation factor, because the bacteriophage MS2 coat protein and the spliceosomal U1A protein, which are not involved in eukaryotic translation, can also repress translation when cognate binding sites are introduced into reporter mRNAs (Stripecke & Hentze, 1992; Stripecke et al., 1994). A cap-proximal position of the IRE or other repressor protein binding sites is important for translational regulation, because cap-distantly (>60 nt) located sites are functionally impaired (Goossen & Hentze, 1992). In vitro, the translation of IRE-containing mRNAs lacking poly(A) tails is repressed by IRP binding (Walden et al., 1988; Brown et al., 1989; Gray et al., 1993; Kim et al., 1995), and IRP-mediated translational control in vivo occurs without notable changes in the mobility of IRE-containing mRNAs on northern blots (Aziz & Munro, 1986; Rouault et al., 1987), suggesting the lack of involvement of the poly(A) tail. Because the analysis of IRE-controlled mRNAs has been limited to northern blots (Aziz & Munro, 1986; Rouault et al., 1987), we applied a more directed and sensitive technique (Sallés et al., 1994; Sallés & Strickland, 1995) to examine the poly(A) tail of ferritin H- and L-chain mRNAs. Unexpectedly, these experiments revealed regulated changes in poly(A) tail length. We characterize this novel mechanism.

RESULTS

IRE functions as a conditional polyadenylation regulatory element

To investigate whether translational regulation by IRPs affects poly(A) tail length, we employed PCR-based poly(A) test [PAT] described by Strickland and coworkers (Sallés et al., 1994; Sallés & Strickland, 1995). Essentially, the combination of an mRNA-specific primer with a poly(A) tail anchor primer allows estimation of the lengths of the poly(A) tails of specific mRNAs (Fig. 1A). The PAT assay was validated with in vitro-synthesized mRNAs containing poly(A) tails of exactly 51 or 96 adenosines (see Materials and Methods). In each case, the major amplification products had the correct lengths (± approximately 10 nt) (Fig. 1B). In addition, minor amplification products of smaller size were generated; the correct lengths of the major amplification products were confirmed independently by comparison with a DNA sequencing reaction (data not shown).

HeLa cells were transfected with human growth hormone (hGH) reporter constructs bearing a cap-proximal IRE (F17), a cap-distant IRE (F64), or no IRE (D4GH) (Fig. 2). The transfected cells were grown for 2 h in the presence of ^{35}S-methionine and harvested. One half of the cells from each transfection was analyzed for hGH biosynthesis by immunoprecipitation, the other half was used for the preparation of poly(A)-enriched RNA. Consistent with our previous findings (Goossen & Hentze, 1992), IRP binding represses hGH biosynthesis in cells treated with the iron chelator desferrioxamine (D) from F17 mRNA, but not from F64 or D4GH mRNAs (Fig. 3A). When IRP binding to the IREs is switched off by treatment with the iron source, hemin (H), F17 mRNA is translationally derepressed. The northern blot gives no indication of size heterogeneities between the different hGH mRNAs and shows that more F17 mRNA was produced than F64 or D4GH mRNAs (Fig. 3B), probably due to small variations in transfection efficiencies. Therefore, hemin addition does not enhance the translation efficiency (protein synthesis per unit mRNA) of F17 mRNA over that of F64 or D4GH mRNAs, consistent with previous findings (Goossen & Hentze, 1992). Analysis of the cellular mRNAs by the PAT assay shows that the unregulated D4GH mRNAs bear a poly(A) tail of ~100–330 nt in iron-deficient and in iron-replete cells (lanes 5 and 6). By contrast, the poly(A) tails in F17 mRNA also consist of ~100–330 nt in iron-replete cells, but are reduced to ~50–270 adenosines in iron-deficient cells, where these transcripts are translationally repressed (compare lanes 1 and 2). Interestingly, no alteration in poly(A) tail length is seen for F64 mRNAs (lanes 3 and 4). Because the IRE in F64 mRNA binds IRP-1 with similar affinity as that of F17 mRNA, at least in vitro (Goossen & Hentze, 1992), without being translationally



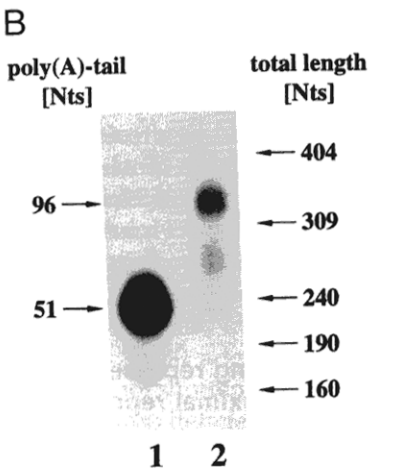


FIGURE 1. **A:** Schematic representation of the PCR-based poly(A) test (PAT). Oligo(dT) primers (12–18mers) are annealed at 42 °C to 3–5 ng of poly(A)-enriched or total cytoplasmic RNA and ligated. At the termini of the poly(A) tail, a short poly(A) stretch remains (marked by arrows) due to the instability of short dT/A hybrids at 42 °C. A fivefold molar excess of TTT-anchor (GC-rich) over oligo(dT) is added to the reaction, annealed, and ligated to the 5′ end of the poly(dT) primer at 12 °C. The low temperature allows the annealing of oligo(dT) to the remaining adenosines on the poly(A) tail, whereby the molar excess of TTT-anchor favors its annealing over the oligo(dT). The ligated poly (dT) is used to prime reverse transcription. The resulting cDNA serves as a template for PCR using α-^{32}P dATP, an mRNA specific primer, and the TTT-anchor primer. The range of PCR products reflects the size distribution of the poly(A) tails. **B:** Validation of the PAT assay using mRNAs with known poly(A) tail lengths as templates. In vitro-synthesized mRNAs, containing a poly(A) tail of either 51 (lane 1) or 96 (lane 2) adenosines, were evaluated by the PAT assay. The sizes of the PCR fragments were determined in comparison to a ^{32}P-labeled *Msp* I-digested pBR322 or in comparison to a DNA sequencing reaction (not shown). The poly(A) tail lengths are calculated by subtraction of the number of nucleotides between the first nucleotide of the mRNA-specific primers and the first adenosine of the poly(A) tail from the size of the PCR fragment and subtraction of the 18 nt derived from the G/C-rich sequence of the oligo(dT)-anchor. Poly(A) tail length is indicated on the left. Fragment sizes of the standard are indicated on the right.

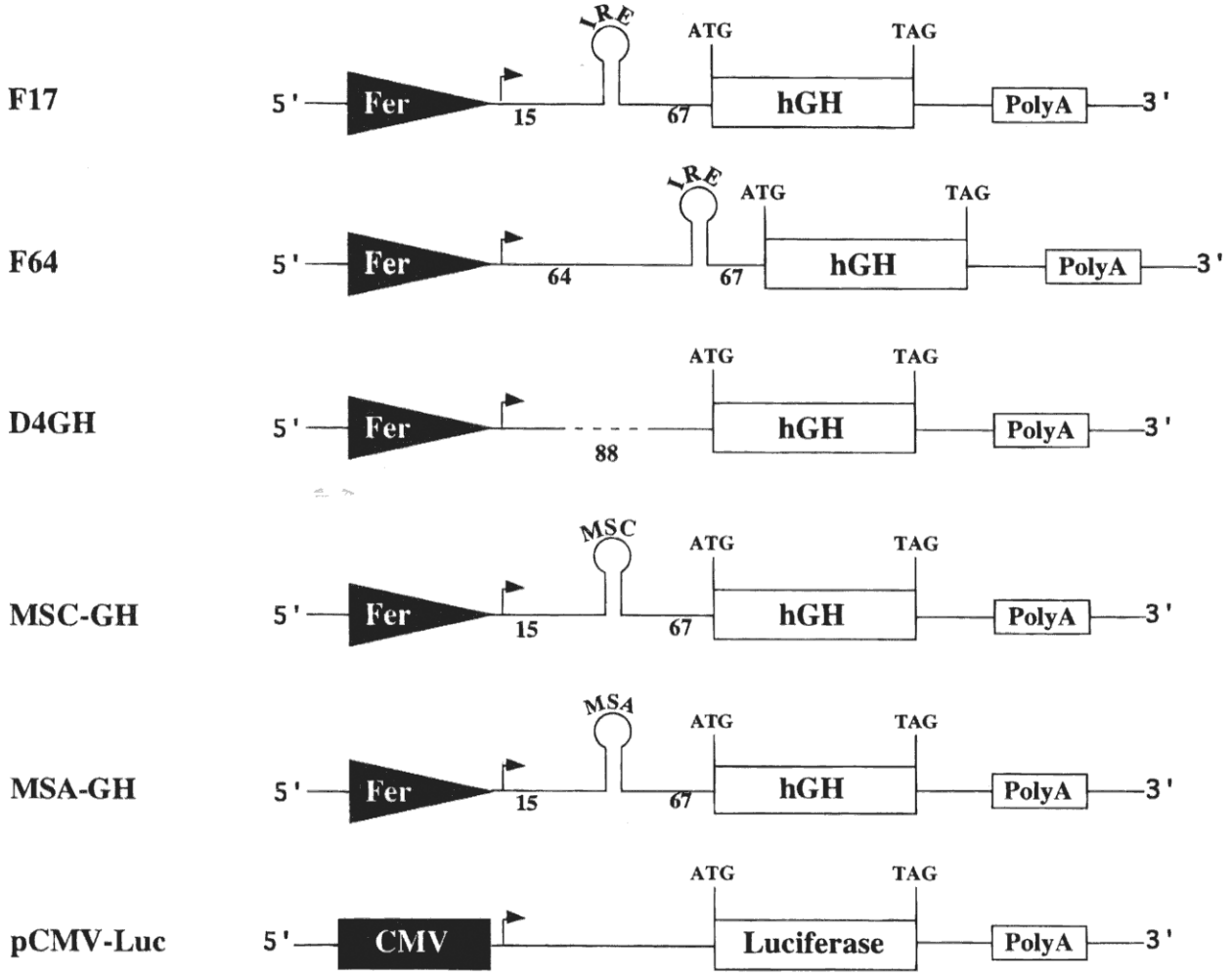


FIGURE 2. Reporter and control plasmids used in HeLa cell transfection experiments. The transcription of the human growth hormone (hGH) reporter mRNAs is driven by the human ferritin H-chain promoter (Fer); the poly(A) signal is derived from the hGH gene (DeNoto et al., 1981), the luciferase transfection efficiency control plasmid pCMV-Luc by the cytomegalovirus (CMV) early promoter. The RNA sequences of the protein binding sites (IRE, MSC, and MSA) are predicted to form stem-loop structures and are described in the references given in Materials and Methods. Nucleotide distances between the transcription start site, the protein binding, and the translation start sites are indicated.

repressed (Fig. 3A; Goossen & Hentze, 1992), this finding indicates that poly(A) tail shortening requires translational repression rather than just IRP binding. As expected, the poly(A) tails of the co-transfected luciferase mRNAs remained unaffected by iron perturbations (data not shown). We conclude that the IRE acts as a polyadenylation control element in vivo. Moreover, polyadenylation control is intimately coupled with IRE function as a translational regulatory element and not solely as an IRP binding site.

Poly(A) tail shortening represents a general response to cap-proximal repressor proteins

The results obtained with the IRE-regulated hGH reporter mRNAs demonstrate that the poly(A) tail is shortened when translation is repressed by a cap-proximal IRE/IRP complex. Poly(A) tail shortening could be induced specifically by the IRE/IRP complex or represent a more general consequence of translational repression by cap-proximal repressor proteins. To distinguish between these two possibilities, experiments were designed in which mRNA translation is repressed sterically by a complex between the bacteriophage MS2 coat protein and a cap-proximal high-affinity binding site (MSC) (Stripecke & Hentze, 1992; Stripecke et al., 1994). MSC-GH, or a point-mutated version that displays drastically reduced affinity for the MS2 coat protein (MSA-GH), were co-transfected into HeLa cells with an MS2 coat protein (MS2-CP) and a luciferase expression plasmid. Translational repression by MS2-CP binding to MSC-GH mRNA is specific, because translation of MSC-GH but not MSA-GH mRNA is repressed in the presence of the MS2 coat protein (Fig. 4A). Importantly, MS2-CP causes shortening of the poly(A) tail of MSC-GH, but not

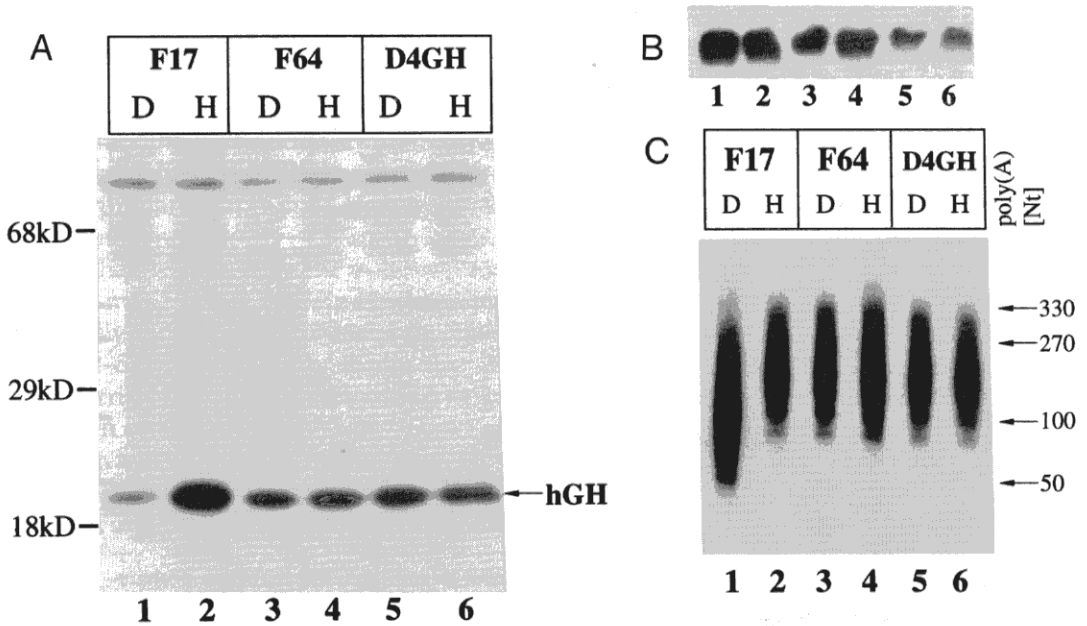


FIGURE 3. IRE functions as a polyadenylation control element. Human growth hormone (hGH) reporter plasmids bearing a cap-proximal IRE (F17, lanes 1 and 2), a cap-distant IRE (F64, lanes 3 and 4), or no IRE (D4GH, lanes 5 and 6) were transiently transfected into HeLa cells. pCMV-Luc was cotransfected as control. Transfected cells were treated either with the iron chelator desferrioxamine (D), or hemin (H) as an iron source and labeled subsequently for 2 h with [^{35}S] methionine. Half of the cells were used for the preparation of protein extracts, the other half for the preparation of mRNA. **A:** Cell extracts were analyzed by quantitative growth hormone immunoprecipitations. The position of the hGH protein is indicated, the position of size standards is indicated on the left. **B:** Northern blot analysis of poly(A)-enriched RNA using a random prime labeled DNA probe specific for hGH. **C:** PAT analysis. The estimated length of the poly(A) tail is indicated on the right. It is calculated by subtraction of the number of nucleotides between the mRNA-specific primer and the poly(A) addition site, 14 nt 3′ of the poly(A) signal (DeNoto et al., 1981) from the size of the PCR fragment. Poly(A) tail length is 50–270 nt (lane 1) and 100–330 nt (lanes 2–6). PCR fragments were analyzed on a 5% nondenaturing polyacrylamide gel in comparison to the ^{32}P-labeled molecular weight marker VI (Boehringer). The identity of the PCR products was confirmed by cloning and subsequent sequence analysis.

MSA-GH mRNA (Fig. 4C, compare lanes 1 and 3) without apparent changes in hGH mRNA quantity or mobility on a northern blot (Fig. 4B). Repression by MS2-CP induces a similar reduction in poly(A) tail length of hGH mRNA as IRP-induced repression (compare Figs. 3C and 4C). The luciferase control displayed no effect of the expression of the MS2 coat protein on poly(A) tail length (data not shown). Because the MS2 coat protein did not evolve to regulate mRNA translation or cytoplasmic polyadenylation in eukaryotic cells, the data argue that poly(A) shortening reflects a more general consequence of translational repression induced by cap-proximal translational repressor proteins.

Poly(A) tail shortening is associated with but not required for translational repression

From the analysis of the hGH reporter mRNAs, we proceeded to examine the poly(A) tails of endogenous ferritin L- and H-chain mRNAs. Both messages harbor IREs in their 5′UTRs and represent physiological substrates for translational repression by IRPs (Aziz & Munro, 1987; Hentze et al., 1987b). As confirmed in Figure 5A, ferritin L- and H-chain synthesis is equally regulated in response to changes in iron availability. Translational regulation of ferritin L-chain mRNA is accompanied by a profound change in poly(A) tail length from 150–300 adenosines in iron replete to 30–80 residues in iron deficient cells (Fig. 5B). In this experiment, translational repression of L-chain mRNA by IRE/IRP complexes therefore causes poly(A) tail shortening by approximately 100–200 nt. In similar experiments, the poly(A) tails of ferritin L-chain mRNAs varied between 150 and 250 adenosines in iron replete cells, with iron chelator-induced partial deadenylation of more than 50 adenosines and preservation of a poly(A) tail of at least 50 nt (data not shown). These results confirm the findings with F17 mRNA (Fig. 3C) and reveal the previously unnoticed control of polyadenylation as part of the translational regulation of ferritin L-chain mRNA. In contrast to the L-chain mRNA, ferritin H-chain mRNA bears a rather short poly(A) tail of only 50–80 adenosines, even in iron replete cells, which is not shortened further when translation is repressed in iron deficient cells (Fig. 5C). Similar results were obtained with total cytoplasmic RNA instead of oligo-dT sepharose-selected material (data not shown). These results imply that poly(A) shortening is not required for IRP-mediated translational re-

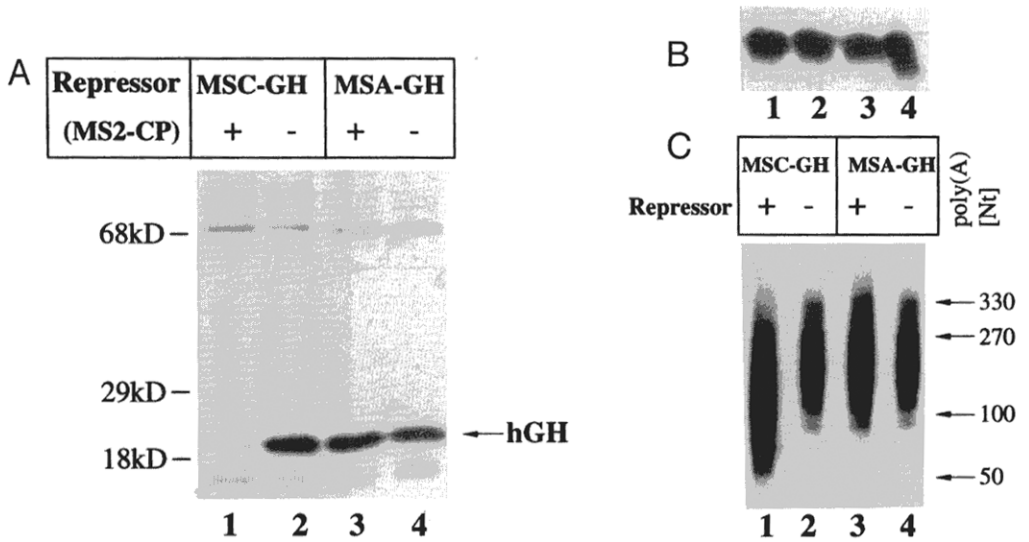


FIGURE 4. Translational repression represents a general response to cap-proximal repressor proteins. hGH reporter plasmids bearing the high-affinity binding site for the MS2 coat protein (MSC-GH; lane 1 and 2) or a mutated binding site (MSA-GH; lane 3 and 4) were transiently transfected into HeLa cells, either in the presence of the MS2 coat protein (MS2-CP) expressing plasmid pSG5-CP (lane 1 and 3) (Stripecke et al., 1994) or the pSG5 vector (lane 2 and 4). pCMV-Luc was cotransfected as a control. **A:** Cell extracts were analyzed by quantitative growth hormone immunoprecipitation. The position of the hGH protein is indicated on the right, the position of size standards is indicated on the left. **B:** Northern blot using a radiolabeled probe for hGH mRNA. **C:** PAT analysis. PCR fragments were analyzed on a 5% nondenaturing polyacrylamide gel. The calculated length of the poly(A) tail is indicated on the right.

pression and add a strong argument in favor of the notion that translational control exerted at the 5′ end of the mRNA can cause (but does not require) changes in 3′ end polyadenylation.

Effect of ribosome association on poly(A) tail length

Under conditions of complete translational repression by IRP-1, ribosomes cannot associate with the mRNA because IRP-1 imposes a block at an early step in translation *initiation* (Gray & Hentze, 1994). Translation *elongation* can be blocked pharmacologically in such a way that mRNAs remain associated with ribosomes (e.g., cycloheximide, anisomycin) or are released (e.g., puromycin) (Allen & Zamecnik, 1962; Westerberg et al., 1976). Verrucarin inhibits early steps of translation and induces the "run off" of bound ribosomes (Wei & McLaughlin, 1974; Cannon, 1976; Fresno & Vázquez, 1979). To gain further insight into the mechanism(s) underlying the regulation of poly(A) tail length, we tested the effects of these translation inhibitors on

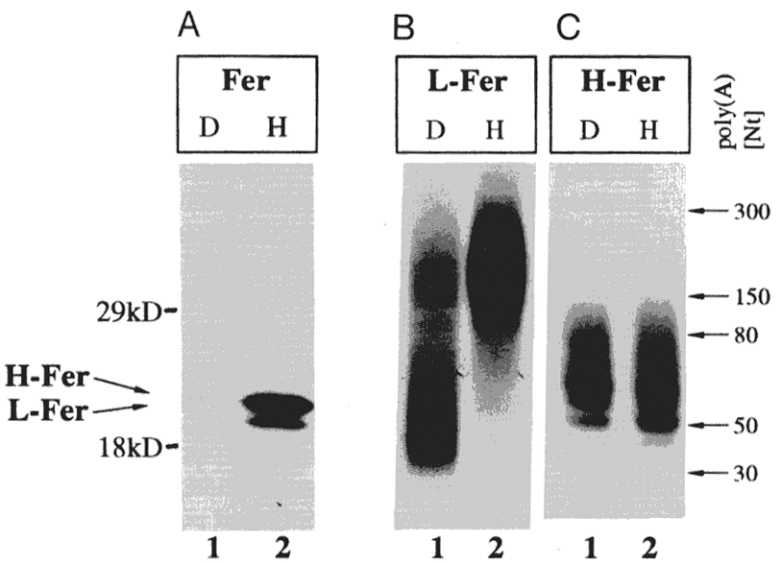


FIGURE 5. Poly(A) tail shortening is associated with but not required for translational repression. Protein extracts and poly(A)-enriched mRNA are the same as in Figure 3. **A:** Cell extracts were analyzed by quantitative ferritin immunoprecipitation. The positions of ferritin H-chain (H-Fer; upper band) and L-chain (L-Fer; lower band) are indicated. Marker positions are given on the left. **B:** PAT of ferritin L-chain mRNA. **C:** PAT of ferritin H-chain mRNA. The calculated length of the poly(A) tails is indicated on the right.

poly(A) tail length of L-ferritin mRNAs (Fig. 6). HeLa cells were either left untreated (U) or were treated with cycloheximide (C), anisomycin (A), puromycin (P), or verrucarin (V) for 12 h; for the last hour of treatment, ^{35}S-methionine was added to monitor the effectiveness of the inhibitors. After harvesting, protein extracts were prepared from half of the cells for analysis of protein synthesis by ^{35}S incorporation (Fig. 6A), the other half was used for the isolation of total RNA. As shown in Figure 6A, protein synthesis was inhibited profoundly by all four antibiotics (compare lane 1 with lanes 2–5). The PAT assay shows that the poly(A) tails of L-ferritin mRNAs were shortened by approximately 70 nt in cells treated with the "release inhibitors" puromycin and verrucarin (Fig. 6B, lanes 4 and 5) when compared to untreated control cells (Fig. 6B, lane 1). By contrast, in cells treated with cycloheximide or anisomycin, the poly(A) tails of L-ferritin mRNAs remain unchanged in comparison to the untreated control (Fig. 6B, compare lanes 1–3). Some poly(A) tail shortening was observed when 50 times higher concentrations of cycloheximide (50 μg/ml) were used (data not shown). At this concentration, cycloheximide can prevent the binding of ribosomes to mRNAs (Hogan, 1969). A difference exists between the "deadenylation patterns" of L-ferritin mRNA in the presence of antibiotics (Figs. 6, 7) and by iron perturbations (Fig. 5). The effect of antibiotic inhibitors on poly(A) tail length of L-ferritin mRNA is compared to the poly(A) tail length in non-iron perturbed control cells. In these controls, ferritin mRNA is not fully derepressed, as it is after hemin treatment. Therefore, the poly(A) tails of ferritin L-chain mRNAs in hemin treated cells are longer than those in the non-antibiotic treated controls.

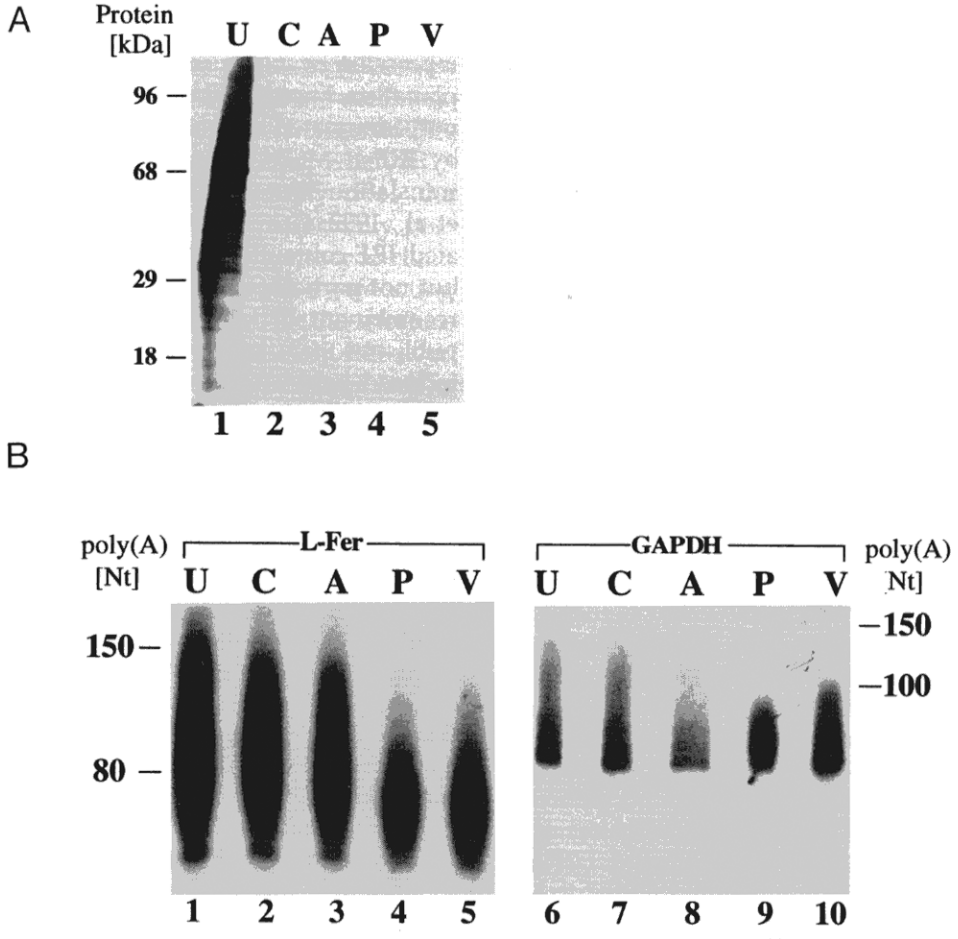


FIGURE 6. Inhibition of protein synthesis by antibiotics: Effect of ribosome association on poly(A) tail length. HeLa cells were left untreated (U; lane 1 and 6) or were treated with 1 μg/ml of cycloheximide (C; lane 2 and 7), 5 μg/ml anisomycin (A; lane 3 and 8), 2.5 μg/ml of puromycin (P; lane 4 and 9), or 5 μg/ml verrucarin (V; lane 5 and 10) for 12 h. For the last hour, cells were grown in the presence of 50 μCi [L-^{35}S] methionine. **A:** Equal amounts of total protein were analyzed for [L-^{35}S] methionine incorporation on an 11% SDS-polyacrylamide gel. The position of size standards is indicated on the left. **B:** Total RNA was analyzed by PAT assay for poly(A) tail length of L-ferritin mRNA (L-Fer; lane 1–5), and GAPDH mRNA (lane 6–10). PCR fragments were analyzed on a 5% denaturing polyacrylamide gel in comparison to the ^{32}P-labeled *Msp* I-digested pBR322-marker (NEB). Poly(A) tail length of L-ferritin mRNA is indicated on the left. Poly(A) tail length of GAPDH mRNA is indicated on the right.

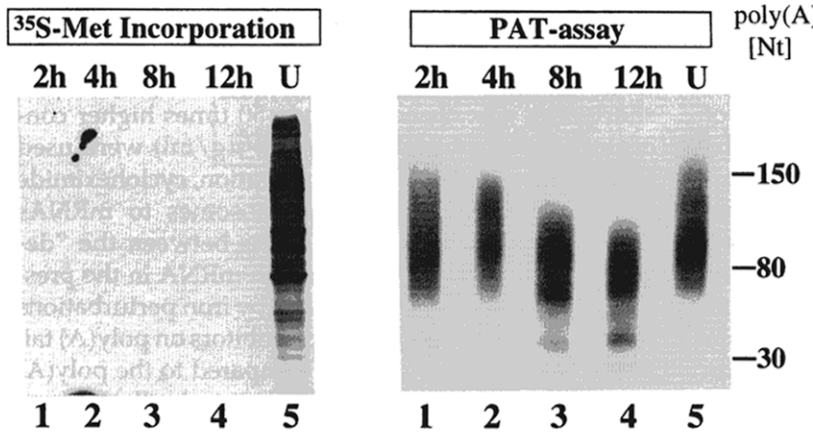


FIGURE 7. Poly(A) tail shortening is kinetically slow. Cells were treated with 5 μg/ml verrucarin for 2 h (lane 1), 4 h (lane 2), 8 h (lane 3), and 12 h (lane 4), or were left untreated (U; lane 5). For the last hour, cells were grown in the presence of [L-^{35}S] methionine. Left: Equal amounts of total protein were analyzed for [L-^{35}S] methionine incorporation on a 11% SDS-polyacrylamide gel. Right: Total RNA was analyzed for poly(A) tail length of L-ferritin mRNA by the PAT assay. PCR fragments were analyzed on a 5% denaturing polyacrylamide gel, loading approximately equal counts of amplification products to assess poly(A) tail length distribution.

The results shown in Figure 6 suggest that lack of ribosome association rather than the lack of ongoing translation per se leads to poly(A) tail shortening. Unless both L-ferritin and transfected hGH mRNAs contain specific sequences that allow regulated poly(A) tail shortening, one would expect that mRNAs that are not translationally regulated by 5'UTR repressor proteins are nevertheless subject to poly(A) tail shortening in response to the pharmacologically induced release of ribosomes. We therefore tested whether glycerine aldehyde phosphate dehydrogenase (GAPDH) mRNA, which is not a known substrate for translational control by a cap-proximal repressor protein, is subject to poly(A) tail shortening when cells are treated with ribosome release inhibitors. GAPDH contains a poly(A) tail of approximately 150 adenosines in untreated HeLa cells (Fig. 6B, lane 6). The poly(A) tail is shortened by approximately 50–70 nt in the presence of puromycin and verrucarin (Fig. 6B, lane 9 and 10), whereas the poly(A) tail remains unchanged in comparison to the untreated control in cycloheximide- or anisomycin-treated cells (Fig. 6B, compare lane 6 with lanes 7 and 8). Thus, ribosome association seems to be a general determinant for poly(A) tail shortening of mRNAs that contain a sufficiently long poly(A) tail. As expected, the poly(A) tail length of H-chain mRNA did not differ significantly from the untreated control in response to the release inhibitors (data not shown).

Poly(A) tail shortening is kinetically slow

To examine the kinetics of poly(A) tail shortening, cells were treated with verrucarin for 2, 4, 8, and 12 h, or were left untreated. ^{35}S-methionine was added to the culture media for the last hour of each time point, and protein extract and total RNA were prepared from the same dish of cells. As seen in Figure 7, protein synthesis was inhibited below the detection level at every time point of verrucarin treatment. In contrast, the appearance of shortened poly(A) tails is delayed for 8–12 h, showing that poly(A) tail shortening in response to verrucarin treatment is a rather slow process.

DISCUSSION

The ferritin L-chain mRNA and the GH reporter mRNAs that are translationally repressed by cap-proximal repressor proteins carry shorter poly(A) tails than their translated counterparts. Poly(A) tail shortening may either represent the cause for translational repression or its consequence. We favor the latter explanation, based on the following arguments. (1) Non-polyadenylated mRNAs are translationally repressed by IRP-1, MS2-CP, and the U1A protein in cell-free translation systems (Walden et al., 1988; Stripecke et al., 1992; Gray & Hentze, 1994). (2) A polyadenylated, IRE-containing mRNA is translationally repressed, but not partially deadenylated, following addition of recombinant IRP-1 to rabbit reticulocyte lysate (unpubl. obs.). (3) In transfected cells, deadenylation is induced by MS2-CP binding to a cap-proximal site. Although this interaction can plausibly inhibit 43S complex binding by occupancy of its mRNA entry site, it would appear rather far-fetched to suggest that the bacteriophage protein primarily affects the polyadenylation machinery leading to subsequent effects on mRNA translation. (4) Pharmacological ribosome release inhibitors impose poly(A) tail shortening even on GAPDH mRNA, which is not a known substrate for cap-proximal repressors. (5) Cellular ferritin H-chain mRNAs are subject to translational control without recognizable changes of poly(A) tail length. On the basis of these considerations, we suggest that the poly(A) tail shortening mediated by cap-proximal repressor proteins is the consequence rather than the cause of translational repression.

Regarding the function of translational regulatory proteins that bind to 5'UTRs of mRNAs, at least two different mechanisms for regulation appeared to exist: one involving the translational control element (TCE), which regulates translation and polyadenylation during *Drosophila* spermatogenesis (Schäfer et al., 1990), the other exemplified by the IRE, which controls mRNA binding of the small ribosomal subunit (Gray & Hentze, 1994) without apparent involvement of the poly(A)

tail. As shown in this paper, the previously assumed discrepancy between the TCE and the IRE regarding the regulation of polyadenylation does not exist. This suggests the possibility that a TCE-binding protein(s) may act in a mode akin to IRPs as a repressor of 43S pre-initiation complex association with the *Drosophila* mRNAs. Although TCE-binding proteins have been identified (Kempe et al., 1993), their role as translational regulatory proteins awaits biochemical demonstration. Interestingly, the function of the TCE is also position-dependent (Schäfer et al., 1990), a feature that it shares with the IRE and steric translational regulators (Goossen et al., 1990; Goossen & Hentze, 1992; Stripecke & Hentze, 1992; Gray & Hentze, 1994).

Given that the translational regulation of ferritin mRNAs in somatic cells has been a focus of intensive investigation for more than a decade, it is surprising that the effect on the poly(A) tail has been missed so far. In part, this can be attributed to the analysis of ferritin mRNAs by northern blotting, which has a limited capacity to identify changes in mRNA length, as well as to the reconstitution of ferritin regulation in cell-free systems using nonadenylated templates, and to the focus on ferritin H-chain mRNA by some investigators. As shown in Figure 5C, ferritin H-chain mRNA is not subject to poly(A) tail shortening when IRP-binding suppresses its translation. Why does the H-chain mRNA escape this effect? It is formally possible that *cis*-acting elements present in either H-chain mRNA inhibit deadenylation, or alternatively, *cis*-acting elements present in L-chain mRNA allow the shortening of the poly(A) tail. We find this unlikely, however. Closer analysis of F17 and MSC-GH mRNAs as well as ferritin L-chain mRNA reveals that translational repression leads to *partial* deadenylation, which preserves a poly(A) tail of >30–50 nt. Because the poly(A) tail of H-chain mRNA does not exceed this length significantly even when the message is translated efficiently, the mRNA may not undergo partial deadenylation even when exposed to the deadenylation activity. Moreover, hGH mRNA, which does not appear to have evolved to be regulated specifically by cap-proximal repressors, undergoes regulated poly(A) tail changes when translationally controlled by IRPs or MS2-CP binding. This regulation of hGH mRNA makes it rather unlikely that specific *cis*-acting elements (other than the repressor binding sites) mediate the poly(A) tail regulation. The only requirement appears to be the presence of a sufficiently long poly(A) tail in the translationally active state. The *cis*-acting sequences that specify the differences between the lengths of the poly(A) tails of the ferritin H- and L-chain mRNAs in the translationally active state are not apparent, because both messages possess canonical AAUAAA polyadenylation elements. The protection of a poly(A) tail of 30–70 adenosines may be important to preserve the stability of the repressed mRNAs (Shyu et al., 1991; Decker & Parker, 1993). Indeed, as evident from Figures 3 and 4, the differences in poly(A) tail lengths identified in this study do not appear to affect mRNA stability, demonstrating a lack of obligatory coupling between (partial) deadenylation and mRNA degradation, and suggesting that the observed differences in poly(A) tail lengths are not the result of differences in the stability of the mRNAs with longer and shorter tails.

How do cap-proximal repressors induce poly(A) tail shortening? A formal possibility is that the 5′ repressor protein stimulates a deadenylase directly [in analogy to the stimulation of polyadenylation by 3′UTR CPE-binding proteins (Hake & Richter, 1994)]. This possibility does not, however, represent an attractive model, considering that IRP-binding to F17 mRNA would have to stimulate the deadenylase over a distance of several hundred nucleotides, whereas IRP-binding 47 nt further downstream (F64) would not. Moreover, one would have to postulate that the bacteriophage MS2 coat protein could stimulate the deadenylase activity specifically when binding to MSC-GH mRNA. Nuclear mechanisms such as alterations in transcription rates and thus the regulation of the de novo synthesis of mRNAs with long poly(A) tails or the regulation of polyadenylation in the nucleus are also rather unlikely explanations of our findings, because F17, F64, and D4-GH, as well as MSC-GH and MSA-GH mRNAs, are expressed from identical promoters and IRPs are cytoplasmic proteins not found in the nucleus. Furthermore, one would have to postulate distinct nuclear effects of the translational inhibitors verrucarin and puromycin versus cycloheximide and anisomycin. A more attractive model posits that translation inhibition caused by repressor protein binding and the subsequent loss of ribosome association leads to poly(A) tail shortening. In favor of this hypothesis is the finding that the binding of IRP to F64 mRNA, which does not cause translational repression, also does not cause poly(A) tail shortening. Cap-proximal repressor complexes, like the IRE/IRP complex, block the binding of the 43S preinitiation complex to the mRNA (Gray & Hentze, 1994) and thus inhibit translation by releasing the mRNAs from polyribosomes (Rogers & Munro, 1987). Experiments using pharmacological translation inhibitors showed that it is this lack of association with ribosomes that leads to the shortening of the poly(A) tail (Fig. 6). Only polyribosome release inhibitors, like puromycin and verrucarin, were able to cause a reduction in poly(A) tail length. Inhibitors of translation that preserve the ribosomal association of mRNAs, like cycloheximide and anisomycin, did not induce any significant changes in poly(A) tail length in comparison to the untreated control cells. This finding is consistent with earlier reports, in that translation inhibition by cycloheximide did not alter the polyadenylation states of transcripts (Brawerman, 1973, 1981; Merkel et al.,

1976). Because all four antibiotics inhibit protein synthesis, the lack of ribosome association rather than the lack of ongoing translation appears to constitute an important determinant for the induction of poly(A) tail shortening.

A relationship between ongoing translation and poly(A) tail length has also been observed in several other studies. Muhlrad et al. (1995) have shown in yeast that inhibition of translation by insertion of a strong secondary structure into the 5'UTR of the stable PGK1 transcript leads to a three- to fourfold enhanced rate of deadenylation. In contrast to our findings in mammalian cells, the faster deadenylation rate greatly stimulated the decay of the PGK1 transcript. On the contrary, the poly(A) shortening and subsequent degradation of the unstable c-fos and c-myc mRNAs in mammalian cells is slowed down when translation is inhibited (Laird-Offringa et al., 1990; Schiavi et al., 1994). These findings differ from those reported here, where partial deadenylation results from a change in ribosome association without affecting the degradation of stable mRNAs.

Consistent with our interpretation that partial deadenylation is a consequence rather than the cause of altered translation, the kinetics of repressor-induced as well as verrucarin-induced deadenylation are slow. These slow kinetics have so far impeded our attempts to assess the reversibility of deadenylation. Cells treated with the iron chelator desferrioxamine or ribosome release-promoting antibiotics for 8–12 h to induce deadenylation would have to be treated subsequently with an iron source [or the antibiotic must be effectively removed] and a transcription inhibitor to prevent the de novo synthesis of fully adenylated mRNAs. Such combinations of chemicals for many hours are highly cytotoxic. Alternative approaches must be devised to investigate whether re-adenylation occurs when the translational block is removed, and whether re-adenylation precedes or follows the re-initiation of translation. Such experiments should also help to investigate the functional role of repression-induced partial deadenylation, perhaps as a means to support the maintenance of a translationally repressed state (see below).

Several models can be envisaged for how translational repression of mRNAs results in a shorter poly(A) tail length in comparison to translated mRNAs. In principle, the mRNA could be deadenylated specifically when the repressor protein binds or actively adenylated when it associates with polyribosomes. Active "unbalanced" adenylation of translated mRNAs appears relatively unlikely in the light of the shorter poly(A) tails of polysomal compared to nuclear mRNA (reviewed in Brawerman, 1981). We favor the model depicted in Figure 8. This model views the length of the poly(A) tail in the cytoplasm as the result of the association of a messenger RNA with polyribosomes or the mRNP. Ribosome association would favor longer poly(A) tails (or diminished deadenylation compared to the nuclear precursor), whereas a translationally repressed mRNA would be subject to enhanced deadenylation. Thus, the translation state of an mRNA would modulate its poly(A) tail length. At the moment, we cannot distinguish whether this results from ribosomal protection of the poly(A) tail from deadenylases or the presence of specific deadenylases in mRNPs. An implication of this model is that translational repressors other than the cap-proximal ones investigated here may induce similar responses. Because the poly(A) tail acts as an enhancer of translation initiation, the partial deadenylation of the mRNA under conditions when its translation is required to be repressed could help to sustain the translationally repressed state.

MATERIALS AND METHODS

Construction of recombinant plasmids

The construction of the hGH reporter plasmids and the MS2-coat protein expression plasmid used for the HeLa cell transfections was described previously: F17 (also called Fer-GH) (Dandekar et al., 1991); F64 (Goossen & Hentze, 1992); D4GH (Hentze et al., 1987a); MSC-GH, MSA-GH, and pSG5-CP (Stripecke et al., 1994). pCMV-Luc, which contains the CMV promoter, the luciferase open reading frame, the SV40-t-intron, and the SV40 late polyadenylation site was a kind gift of Dr. Vivanco Ruiz (European Molecular Biology Laboratory, Heidelberg, Germany). For the construction of p96A, a blunt-ended *Eco*R I/*Bam*H I fragment from T3pA (Iizuka et al., 1994) was introduced into the blunt-ended *Pst* I site of pI-12.CAT (Stripecke & Hentze, 1992; Gray et al., 1993). To construct p51A, complementary synthetic oligodeoxyribonucleotides, containing 51 adenosine residues were inserted into the *Bam*H 1/*Eco*R 1 site of PLT-1, a pGEM3Zf+ plasmid containing part of the preprolactin open reading frame.

Transient transfection, metabolic labeling, and immunoprecipitation

HeLa cells were maintained at 37 °C/5% CO_2 in Dulbecco modified Eagle medium containing 10% fetal calf serum and 1 U/ml of penicillin/streptomycin (GIBCO), and transfected by the calcium phosphate method (Graham & van der Eb, 1973). Fifteen micrograms of hGH reporter plasmids, 1.2 μg of pSG5-CP (or pSG5 vector alone), and 2 μg of pCMV-Luc were co-transfected as indicated in the figure legends. Sodium butyrate (5 mM, pH 7.3), as well as 100 μM hemin or 100 μM desferrioxamine, were added ~24 h after transfection. Following a 12-h incubation, the cells were labeled for 2 h with 50 μCi of [L-^{35}S] methionine (specific activity, 1,300 Ci/mmol) in 2.5 ml of methionine-free medium, washed twice with ice-cold buffered saline, and harvested. Half of the cells were used for RNA extraction (see below), the other half for the preparation of a cellular lysate in 1 ml of ice-cold buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% phenylmethylsulfonyl fluoride, 10 μg of leupeptin per mL) by vortexing and incubation on ice for 30 min.

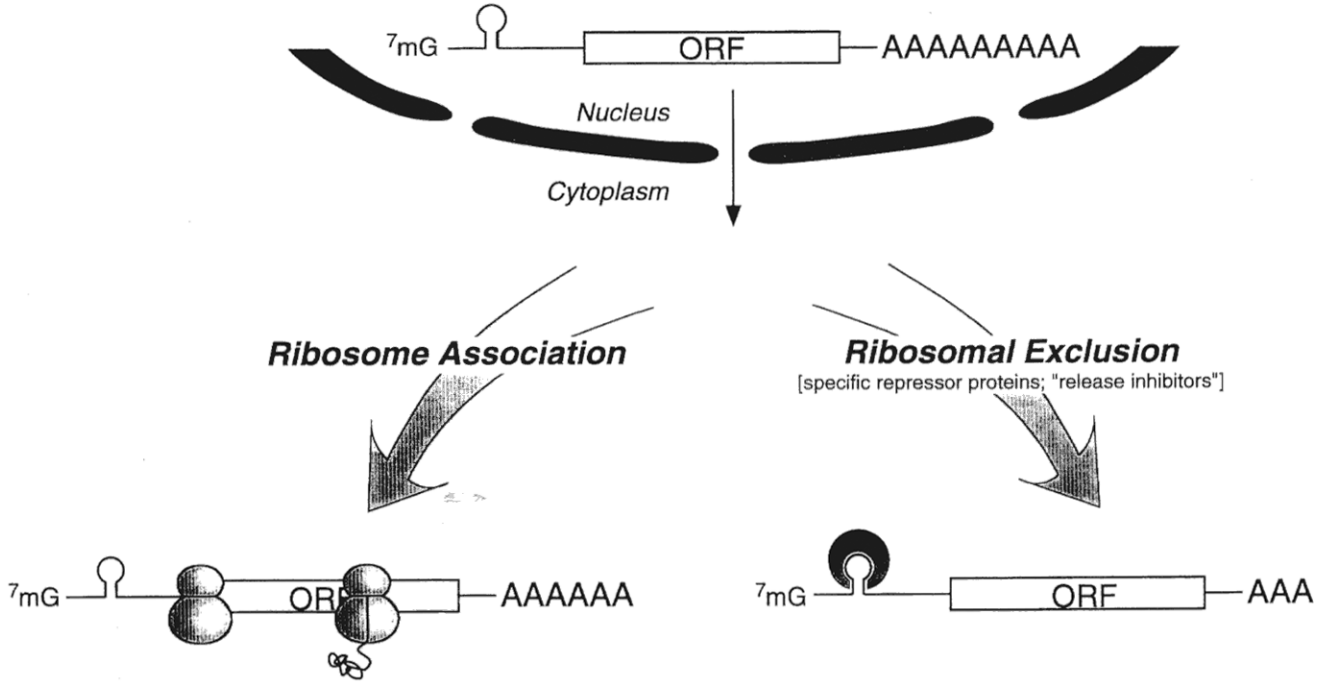


FIGURE 8. Regulated poly(A) tail length and the role of ribosome association. mRNAs containing a cap-proximal repressor protein binding site are polyadenylated in the nucleus and transported to the cytoplasm. The mRNAs are either translated or translationally repressed by the binding of the repressor protein, which blocks the association of the mRNA with ribosomes. We suggest that ribosome association (normally because of ongoing translation) favors the maintenance of longer poly(A) tails, whereas exclusion from ribosomes into the mRNP by binding of translational repressors (or experimentally added antibiotics) predisposes the mRNAs to pronounced poly(A) tail shortening.

The protein lysate was centrifuged at 15,000 × *g* for 5 min at 4 °C, and equal aliquots of supernatant (1×10^7 cpm, normalized for trichloroacetic acid-insoluble radioactivity) were used for immunoprecipitation with saturating quantities of polyclonal anti-hGH (National Hormone and Pituitary Program, Baltimore, Maryland) or anti-ferritin (Boehringer, USA) antibodies using protein A-Sepharose. The gel was washed twice (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100), boiled in electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.7 M 2-mercaptoethanol, 0.05% bromophenol blue), and analyzed on a 15% SDS-polyacrylamide gel. Luciferase assays from total protein lysates were performed as described previously (Brasier et al., 1989).

Treatment of cells with translation inhibitors

Cells were treated for 12 h with 1 μg/ml of cycloheximide, 5 μg/ml anisomycin, 5 μg/ml verrucarin or 2.5 μg/ml of puromycin, and labeled for 1 h with 50 μCi of [L-^{35}S] methionine (specific activity, 1,300 Ci/mmol) in 2.5 ml of methionine-free medium in the presence of inhibitor. Cells were washed twice with ice-cold buffered saline and harvested. Half of the cells were used for RNA extraction (see below), the other half for the preparation of a cellular lysate (see above). ^{35}S-methionine incorporation was determined by analyzing equal amounts of total protein on an 11% SDS-polyacrylamide gel.

Isolation of total RNA and poly(A) enriched mRNA from HeLa cells.

Total RNA was prepared with the RNA-Clean™ system (Angewandte Gentechnologie Systeme GmbH, Heidelberg) according to the manufacturers' instructions. Poly(A)-enriched mRNA was prepared either directly from the cell pellet or from total RNA with the Dynabeads® mRNA DIRECT kit (DYNAL) as described in the handbook.

Northern analysis

Three-hundred nanograms of poly(A)-enriched RNA were resolved electrophoretically in denaturing agarose gels and electrotransferred onto nylon membranes. The RNA was UV crosslinked to the membrane and hybridized to a PCR-amplified, radiolabeled hGH probe.

Synthesis of mRNA with defined poly(A) tail length in vitro

For the synthesis of an mRNA containing 96 adenosines at the 3′ end, p96A was linearized with *Hin*d III, and mRNA was transcribed in vitro according to the manufacturer's instructions using T7 polymerase. Nonadenosine residues and the two terminal adenosines, present at the 3′ terminus of the mRNA due to linearization of the plasmid 14 nt downstream of the 3′ end of the poly(A) tail were removed by adding 100 pmol of an antisense oligodeoxyribonucleotide

with exact complementarity to these 14 nt (5′-CCTAGAGG ATCCCCTT-3′) and RNAseH directly to the in vitro transcription reaction. A transcript containing 51 adenosines at the 3′ end was transcribed from MpH linearized p51A, using SP6 polymerase. Linearization with Mph assured that the poly(A) tail terminates with adenosine residues.

Analysis of poly(A) tail length by PCR

The PCR-based analysis of poly(A) tail length, poly(A) test, or PAT-analysis was performed exactly as described by Sallés and Strickland (1995) and depicted in Figure 1A with the sole exception that 3–5 ng poly(A)-enriched mRNA was used as the input material for PAT cDNA synthesis from the transfected hGH constructs. For the PAT analysis of cellular mRNAs, 50–250 ng total RNA or 3–5 ng poly(A)-enriched mRNA were used as the input material. The PCR products were phenol chloroform extracted, ethanol precipitated with 2.5 M ammonium acetate to remove unincorporated label, and approximately 25% of each reaction was heated to 72 °C for 2 min before separation on a 5% nondenaturing or 5% denaturing polyacrylamide gel along with the ^{32}P-labeled molecular weight marker VI (Boehringer) or a ^{32}P-labeled *Msp* I-digested pBR322 (NEB) and visualized by autoradiography. In general, analysis of the PAT samples on denaturing or nondenaturing polyacrylamide gels yielded similar results.

The mRNA-specific PCR primers were designed to hybridize to the following positions: in hGH mRNA, 177 nt upstream of the polyadenylation signal (5′-ATGGACAAGG TCGAGACATTC-3′); in human L-chain ferritin mRNA, 153 bases upstream of the polyadenylation signal (5′-GGCTCA CTCTCAAGCACGAC-3′); in human ferritin H-chain mRNA, 165 nt upstream of the polyadenylation signal (5′-AGCTAA GCCTCGGGCTAAT-3′); in the 3′UTR of pCMV-Luc, 165 nt upstream of the SV40 late polyadenylation signal (5′-GATG TATAGTGCCTTGACTAG-3′); in GAPDH mRNA, 195 nt upstream of the polyadenylation signal (5′-CATGGCCTCCAA GGAGTAAG-3′); in p98A mRNA, 207 nt upstream of the start of the poly(A) tail (5′-CATCATGCCGTCTGTGATGG-3′); in p51A mRNA, 174 nt upstream of the start of the poly(A) tail (5′-CTGCAGGTCGACTCTAGATG-3′). The oligo(dT)-anchor used had the identical sequence as described by Sallés and Strickland (1995): 5′-GCGAGCTCCGCGGCCGCGT$_{12}$-3′.

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