

mRNA Silencing in Erythroid Differentiation: hnRNP K and hnRNP E1 Regulate 15-Lipoxygenase Translation from the 3' End

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Summary

Although LOX mRNA accumulates early during differentiation, a differentiation control element in its 3' untranslated region confers translational silencing until late stage erythropoiesis. We have purified two proteins from rabbit reticulocytes that specifically mediate LOX silencing and identified them as hnRNPs K and E1. Transfection of hnRNP K and hnRNP E1 into HeLa cells specifically silenced the translation of reporter mRNAs bearing a differentiation control element in their 3' untranslated region. Silenced LOX mRNA in rabbit reticulocytes specifically coimmunoprecipitated with hnRNP K. In a reconstituted cell-free translation system, addition of recombinant hnRNP K and hnRNP E1 recapitulates this regulation via a specific inhibition of 80S ribosome assembly on LOX mRNA. Both proteins can control cap-dependent and internal ribosome entry site-mediated translation by binding to differentiation control elements. Our data suggest a specific cytoplasmic function for hnRNPs as translational regulatory proteins.

Introduction

Genetic control of cell metabolism and differentiation, the cell cycle, and embryonic development involve translational regulatory circuits. Defined regulatory elements located within the 5' or 3' untranslated regions (UTRs) are utilized for translational regulation of specific mRNAs. For example, 5' UTR sequences control amino acid metabolism in *Saccharomyces cerevisiae*, where upstream open reading frames (ORFs) and a regulated eIF2 kinase operate a translation reinitiation mechanism that controls 80S ribosome assembly on the GCN4 ORF (Dever et al., 1992; Hinnebusch, 1994). Iron metabolism in mammalian cells is regulated by a molecular mechanism that controls the binding of the 40S ribosomal subunit to the ferritin mRNA by an iron-sensitive regulatory protein that recognizes a specific 5' UTR element (Gray and Hentze, 1994; Hentze and Kühn, 1996). More commonly, however, the 3' UTR harbors the regulatory sequences. Most examples of this type of regulation

have been found to operate during early development in *C. elegans*, *Drosophila*, *Xenopus*, and mammals (Huarte et al., 1992; Jackson, 1993; Wickens et al., 1993, 1996; Evans et al., 1994; Curtis et al., 1995). In spite of its wide occurrence, the mechanisms underlying 3' UTR-mediated translational regulation are poorly understood.

Erythroid 15-lipoxygenase (LOX) is a key enzyme during erythroid cell differentiation. It can attack intact phospholipids and appears to participate in the breakdown of internal membranes (such as mitochondrial membranes) during the late stages of reticulocyte maturation (Rapoport and Schewe, 1986). LOX activity must thus be temporally restricted. LOX mRNA is transcribed as the most abundant message after globin mRNAs in bone marrow erythroid precursor cells, but is translationally silenced until reticulocytes in the peripheral blood undergo the final steps in maturation (Thiele et al., 1981; Höhne et al., 1988). A CU-rich, repetitive sequence motif in the 3' UTR of LOX mRNA (Fleming et al., 1989; Hunt, 1989), which we will refer to as the differentiation control element (DICE), has been shown to mediate this translational silencing (Ostareck-Lederer et al., 1994). LOX silencing appears not to require alterations in poly(A) tail length, because it can be reconstituted by addition of rabbit reticulocyte proteins eluted from a DICE affinity column to a nonpolyadenylated transcript in a cell-free translation system (Ostareck-Lederer et al., 1994). Here, we report the identification, cloning, and expression of two proteins that bind to the DICE, and demonstrate the specificity and selectivity with which they control the translation of LOX mRNA in vivo and in vitro. We show that they are able to silence not only cap-dependent but also IRES (internal ribosomal entry site)-driven translation via a mechanism that causes the specific inhibition of 80S ribosome assembly.

Results

Identification of the LOX Regulatory Proteins as hnRNPs K and E1

To purify and clone proteins that bind to and regulate LOX mRNA, S100 extract prepared from reticulocytes of anemic rabbits was subjected to affinity chromatography on two different biotinylated RNAs: the DICE from the 3' UTR of rabbit LOX mRNA consisting of a 10-fold repeat of a CU-rich 19 nt motif (10R), or nonregulatory LOX sequences downstream from the repeat (NR) as a specificity control. After extensive washing (Figure 1A, lanes 1 and 3, [w]), the retained proteins were eluted and analyzed by SDS-PAGE. In addition to some minor bands, both columns retain the two prominent polypeptides labeled (n.s.) in Figure 1. Two sharp bands migrating at ~66 kDa and ~43 kDa and a diffuse band of ~48 kDa specifically elute from the DICE resin (compare lane 4 with lane 2). In contrast to the reproducible purification of the proteins migrating at 66 kDa and 43 kDa, the appearance of the 48 kDa band was variable. The eluate from the DICE resin, but not that from the NR control

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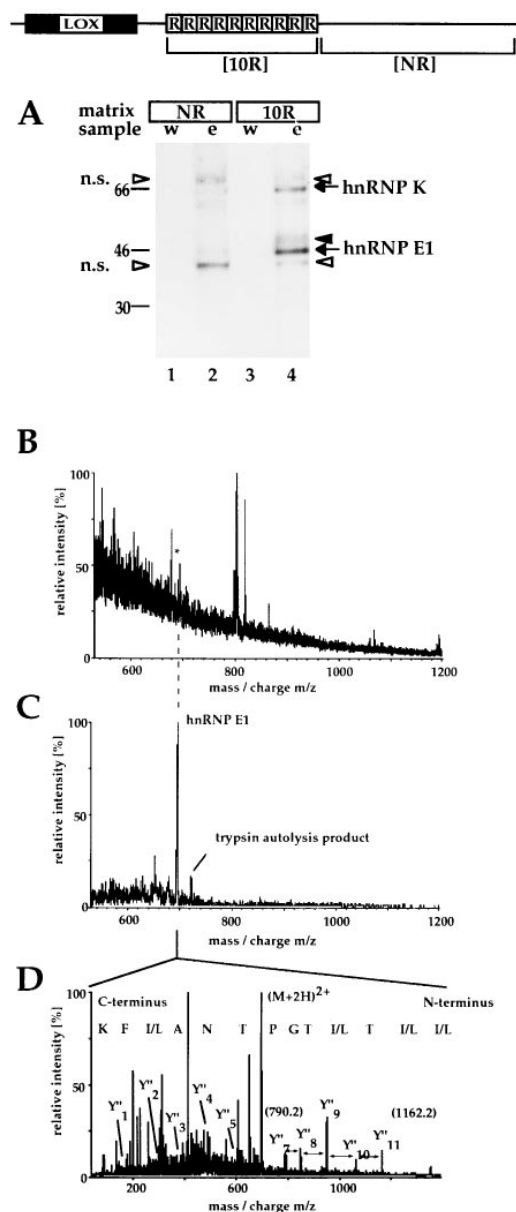


Figure 1. Identification of the LOX Regulatory Proteins as hnRNPs K and E1

Scheme of the LOX mRNA with its 3' UTR-regulatory (10R) and nonregulatory region (NR).

(A) Silver-stained gel with final wash ([w], lanes 1 and 3) and eluates ([e], lanes 2 and 4) after protein purification using NR (lanes 1 and 2) or 10R (lanes 3 and 4) affinity resins. Nonspecific peptides are labeled (n.s.); three bands of 66 kDa, 48 kDa, and 43 kDa specifically elute from 10R.

(B) Nanoelectrospray mass spectrum of the 43 kDa band ([A], lane 4); see Experimental Procedures.

(C) Parent ion scan: detection of ions leading to Ile/Leu-containing peptides.

(D) A sequence tag of a peptide ion identified in (C) was assembled in the high m/z part of the spectrum. Arrows indicate the sequence for peptide sequence tag searching. An alignment of the complete C-terminal ion series is shown.

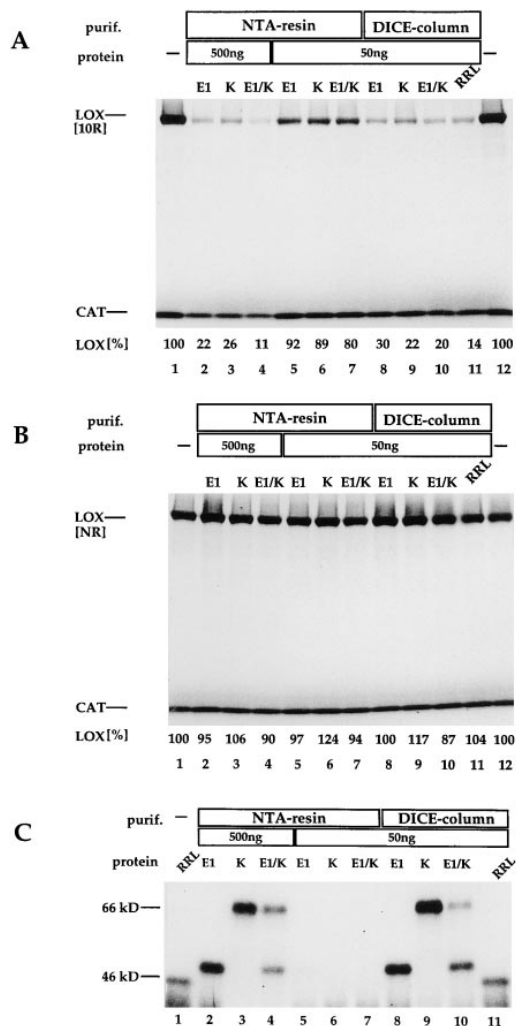


Figure 2. hnRNP E1 and hnRNP K Specifically Regulate LOX mRNA Translation in Rabbit Reticulocyte Lysate

(A and B) LOX-10R ([A], lanes 1–12) or LOX-NR mRNAs ([B], lanes 1–12) were cotranslated with CAT mRNA as internal control. In (A) and (B), dialysis buffer (lanes 1 and 12) or protein fractions purified from NTA or DICE columns were added as indicated, hnRNPs E1/K in a 1:3 ratio. [³⁵S]Met incorporation into translation products was determined by phosphorimaging (Compaq Phosphor Imager with Molecular Dynamics Image Quant software), and the signals in lanes 1 and 12 were taken as 100% standards and averaged. The percentage of LOX expression normalized for the CAT internal control is shown below each lane.

(C) A ³²P-labeled 10R probe was photocrosslinked to the regulatory proteins or to 150 μ g of rabbit reticulocyte lysate (S100) (lane 1) at 254 nm. Note that the N-terminal histidine tag adds $\sim$ 3 kDa to the recombinant proteins.

resin, represses LOX mRNA translation in vitro (data not shown, but see Figures 2A and 5–7), suggesting that the 66 kDa and/or the 43 kDa proteins may represent the regulatory polypeptides.

Both proteins were analyzed by nanoelectrospray mass spectrometry (Wilm et al., 1996a). As shown in Figure 1B for the 43 kDa polypeptide, the affinity preparations yielded protein amounts that were not sufficient for peptides to be distinguished in the mass spectrum.

Parent ion scans of unseparated peptide mixtures (Wilm et al., 1996b) allowed peptide ions to be distinguished from the background (Figure 1C). Peptide sequence tags were derived from the tandem mass spectra. The band at 66 kDa produced 5 peptides, all of which identified the protein as hnRNP K (heterogeneous nuclear ribonucleoprotein K; Matunis et al., 1992; acc. no. S74678). The peptide sequence of the 43 kDa band (Figure 1D) uniquely identified a 13 amino acid peptide from hnRNP E1 (Aasheim et al., 1994; Kiledjian et al., 1995; Leffers et al., 1995; acc. no. X78137). Comparison of the predicted fragment ion masses against the tandem mass spectrum verified the complete sequence. The protein amounts in this small scale isolation were in the low nanogram range, as estimated from the intensity and number of measured peptide ions. The 48 kDa band yielded six peptides that all mapped to the C-terminal part of hnRNP K, suggesting that the variable appearance of this band results from partial proteolysis of hnRNP K during purification. Interestingly, a 48 kDa polypeptide was previously implicated as the LOX mRNA regulatory protein LOX-BP (Ostareck-Lederer et al., 1994). In light of the present data, we believe that LOX-BP corresponds to a 48 kDa fragment of hnRNP K.

DICE-Mediated Translational Control by hnRNP K and hnRNP E1 Is Highly Selective in Cell-Free Extracts and Transfected Cells

To test the function of hnRNPs K and E1 as LOX regulatory proteins, cDNAs were isolated from a HeLa cell library by PCR, sequenced, and expressed as N-terminally histidine-tagged fusion proteins in *E. coli*. Photocrosslinking of recombinant hnRNPs K and E1 demonstrated their specific binding to the DICE (data not shown). In addition to the DICE, poly(C) competed for photocrosslinking of hnRNPs K and E1 to the 10R probe, which was expected from the known relative affinity of both proteins for poly(C) (Swanson and Dreyfuss, 1988; Matunis et al., 1992; Kiledjian et al., 1995).

Translation of LOX mRNA in a rabbit reticulocyte cell-free translation system is specifically silenced by affinity-purified DICE-binding proteins (Ostareck-Lederer et al., 1994). To test whether hnRNP K and/or hnRNP E1 account for this activity, capped LOX mRNA was cotranslated with capped CAT mRNA as an internal specificity control (Figure 2A, lanes 1–12). In the absence of added silencer proteins, both mRNAs are efficiently translated (lanes 1 and 12), whereas the DICE eluate (Figure 1A, lane 4) diminishes LOX translation without significant effect on the translation of CAT (Figure 2A, lane 11). Importantly, recombinant hnRNP E1 (lane 2), hnRNP K (lane 3), or the two combined (lane 4) all silence LOX synthesis. When the DICE is deleted from the 3' UTR, LOX synthesis is unaffected by hnRNPs K and E1 (Figure 2B, lanes 1–12). Thus, silencing by the recombinant proteins is mediated by the DICE. Extraction/re-translation experiments demonstrated that translational silencing is reversible and does not affect the structural or functional integrity of the mRNA (data not shown; see also Figure 6). However, when the recombinant proteins were purified via their histidine tags by NTA chromatography, silencing required approximately 10-fold more

recombinant protein than DICE affinity-purified proteins from rabbit reticulocytes (see Figure 2A; compare lanes 5–7 with lanes 2–4 and lane 11). Secondary purification of the recombinant proteins on a DICE column revealed that only ~5%–10% of the protein could bind. The eluate from the DICE column displays specific repressor activity similar to the affinity-purified material from rabbit reticulocytes (Figure 2A, lanes 8–11), which correlates with its DICE-binding activity (Figure 2C). While these data do not exclude a possible role of additional factors, we conclude that recombinant hnRNPs K and E1 can fully account for translational silencing of LOX mRNA in a cell-free translation system from rabbit reticulocytes.

To investigate whether the complete CU-rich region in rabbit LOX mRNA is required for DICE function, the 10-fold repeat (LOX-10R) was replaced by one (LOX-1R), two (LOX-2R), and four (LOX-4R) copies. While the translation of LOX-1R mRNA was only marginally affected by recombinant hnRNPs K and E1 or the purified reticulocyte proteins, the dose response curves of LOX-10R and LOX-2R mRNAs were similar (data not shown). We also constructed a negative control mRNA of identical length (LOX-2Rmut) in which a central 5' CCCUCUU 3' sequence was replaced by 5' AGAGAGA 3'. This mutant is not silenced by hnRNPs K and E1 (or the cellular proteins) in rabbit reticulocyte lysate (data not shown). Next, the role of hnRNPs K and E1 as 3' UTR-regulatory proteins was examined in transfected HeLa cells. Two luciferase reporter constructs, LUC-2R and LUC-2Rmut, were cotransfected with hnRNP E1 and/or hnRNP K expression vectors. A human growth hormone (hGH) expression vector was cotransfected as an internal specificity control, and an expression vector for U1A (instead of hnRNPs K or E1) was cotransfected as a negative control RNA-binding protein. Luciferase expression was monitored by labeling with [³⁵S]-methionine and immunoprecipitation (Figure 3A) and by enzymatic assay (Figure 3B). While luciferase expression from LUC-2R mRNA is repressed by hnRNP K and hnRNP E1, but not by U1A (Figures 3A and 3B, lanes 1–6), neither the hGH control (Figure 3C), the endogenous ferritin control (Figure 3D), nor the LUC-2Rmut control (lanes 7–12) are affected by either of the two regulatory proteins. Importantly, LUC mRNA levels remain unchanged when both total RNA (Figure 3E) or cytoplasmic RNA (data not shown) are analyzed by Northern blotting. Similar results were obtained in transfection experiments with LUC-10R, using LUC-NR as a negative control (data not shown).

hnRNP E1 has recently been identified as a component of an erythroid ribonucleoprotein complex that binds to a CU-rich region in the 3' UTR of human α -globin mRNA in vitro (α complex) (Kiledjian et al., 1995). Therefore, we evaluated the α -globin 3' UTR in our transfection assay (Table 1). While both hnRNP K and hnRNP E1 silence the translation of LUC-2R mRNA, the α -globin sequence does not function as a DICE in spite of its CU-rich nature. Neither of two negative control RNA-binding proteins (U1A and IRP-1) mimicks the effect of hnRNPs K and E1. Cotransfection of an expression vector for hnRNP A1 affects luciferase activity nonspecifically from LUC-2R, LUC-2Rmut, and LUC- α -globin, possibly due to a general disturbance of RNA metabolism

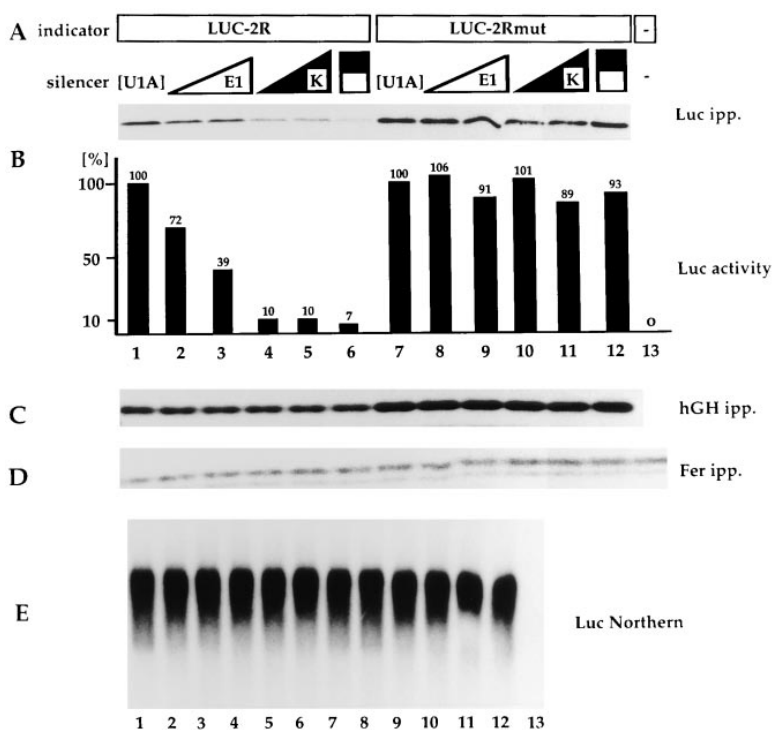


Figure 3. Translational Silencing by hnRNPs K and E1 In Vivo

(A–D) HeLa cells were transiently transfected with 5 μ g of LUC reporter constructs bearing a 38 nt DICE [LUC-2R] (lanes 1–6) or a mutated version as a negative control [LUC-2Rmut] (lanes 7–12), and 5 μ g of a human growth hormone (hGH) internal specificity control plasmid driven by the same promoter as the LUC reporter constructs (lanes 1–12). In addition, 10 μ g of U1A (lanes 1 and 7), 5 μ g (lanes 2 and 8) or 10 μ g (lanes 3 and 9) of hnRNP E1; 5 μ g (lanes 4 and 10) or 10 μ g (lanes 5 and 11) of hnRNP K; or 5 μ g of hnRNP E1 plus 5 μ g of hnRNP K (lanes 6 and 12) expression plasmids were cotransfected. Lane 13 shows an analysis of untransfected cells. Parallel dishes were transfected with the same precipitate, and used for metabolic labeling for 2 hours with [35 S]-Met or for preparation of total RNA and cell extract. Labeled extracts were used for immunoprecipitations with polyclonal antibodies against luciferase (A), hGH (C), and ferritin (D). Unlabeled extracts (50 ng of protein) were used for enzymatic analysis of luciferase activity (B). Luciferase activities in lanes 1 and 7, respectively, were set at 100%. (E) Northern blot of the total RNA hybridized with a luciferase probe. Equal signal intensities were also obtained with a ferritin H chain probe (for lanes 1–13) and an hGH probe (for lanes 1–12, data not shown).

of the transfected cells. We conclude that both hnRNP K and E1 regulate translation with high specificity for the DICE in vitro and in vivo.

Association of LOX mRNA with hnRNP K in Translationally Silent mRNPs of Erythroid Cells

To directly address the role of hnRNPs K and E1 as LOX mRNA regulatory proteins in immature erythroid cells, we isolated translationally inactive mRNPs from reticulocytes of anemic rabbits. To assess the association of specific mRNAs with specific proteins in these mRNPs, hnRNP K-containing complexes were immunoprecipitated with a polyclonal antibody (Figure 4, lanes 4 and 5). As controls, preimmune serum (lanes 2 and 3) or buffer (lanes 6 and 7) was used. RNA was extracted from the precipitates (p) and the supernatants (s), and examined by Northern blotting. Almost half of the LOX mRNA is precipitated by the hnRNP K antibody (ratio of supernatant:precipitate [s:p] = 1.3), whereas barely detectable quantities of LOX mRNA are precipitated when preimmune serum (s:p = 26.0) or buffer (s:p = 38.7) are used. By contrast, the hnRNP K antisera (s:p = 76.0), the preimmune serum (s:p = 53.4), and the buffer control (s:p = 64.2) fail to coprecipitate significant quantities of β -globin mRNA. Due to the lack of an appropriate antiserum, similar experiments with hnRNP E1 could not be performed. The specific association of LOX mRNA with hnRNP K in translationally silenced mRNPs from rabbit reticulocytes supports the role of this protein in the regulation of LOX expression during erythroid differentiation.

Mechanism of Translational Control

As observed in rabbit reticulocyte lysate and in HeLa cells, the regulatory mechanism is also fully operative

in wheat germ extract and meets the same specificity criteria, including the discrimination against the CU-rich element from α -globin mRNA (Figure 5, lanes 15–20), and the lack of silencer activity of the RNA-binding proteins IRP-1 (lane 7) and hnRNP A1 (lane 6) against the DICE. The lack of LOX suppression by recombinant hnRNP A1 *in vitro* is consistent with our interpretation of the data shown in Table 1. Note that recombinant hnRNPs K and E1 purified by DICE affinity chromatography display specific silencing activities similar to the ones displayed by the DICE eluate from rabbit reticulocyte lysate. These results show that no species- or tissue-specific accessory factors are required to enable hnRNPs K and E1 to control translation. To delineate the mechanism of translational control, translation initiation assays were performed. Capped LOX-2R or LOX-2Rmut

Table 1. Functional Discrimination between CU-Rich 3' UTR Sequences from LOX and α -globin mRNAs

Effector	Indicator		
	LUC-2R	LUC-2Rm	LUC- α -globin
U1A	100	100	100
IRP 1	95	101	93
E1	53	86	92
K	40	89	95
E1/K	18	92	93
A1	15	14	12

HeLa cells were transiently transfected as described in the legend to Figure 3. Luciferase activity is expressed as a percentage of the activity from samples cotransfected with 10 μ g of U1A expression plasmid. Luciferase mRNA levels (measured by Northern blotting) were similar in cells transfected with U1A, IRP-1, hnRNP K, and hnRNP E1, but strongly diminished in cells transfected with hnRNP A1.

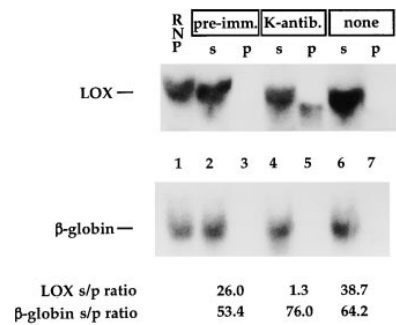


Figure 4. In Vivo Association of hnRNP K and LOX mRNA mRNPs from rabbit reticulocytes were precipitated with hnRNP K polyclonal antiserum (lanes 4 and 5), preimmune serum (lanes 2 and 3) or buffer (lanes 6 and 7). RNA was extracted from mRNPs (lane 1), or supernatants (s) and pellets (p) after immunoprecipitation, and analyzed by Northern blotting with 32 P-dCTP-labeled LOX and β -globin cDNAs. The signals were quantitated by phosphorimaging and expressed as supernatant-to-pellet ratios.

mRNAs were 32 P-labeled, and ribosomes were allowed to assemble during a 5 min incubation in rabbit reticulocyte lysate in the presence or absence of hnRNPs K/E1. The initiation complexes were resolved on 5%–25% linear sucrose gradients and then fractionated, and the association of mRNAs with ribosomal subunits was assessed by scintillation counting of the odd-numbered fractions (Figure 6 graph) and by RNA extraction, gel electrophoresis, and autoradiography of the even-numbered samples (Figure 6 inset). In the absence of the silencer proteins (open circles), both LOX-2R and LOX-2Rmut mRNAs show a biphasic distribution between ribosome-associated (fractions 1–7) and lighter fractions (fractions 10–14). While the hnRNPs K/E1 (indicated by [+]) do not affect ribosome assembly on LOX-2Rmut mRNA (right panel), the association of 80S ribosomes with LOX-2R mRNA is clearly diminished and the transcript shifted to the lighter fractions (left panel). This result demonstrates that the regulatory proteins inhibit translation initiation by preventing 80S ribosome assembly, and confirms that the stability of LOX mRNA is not affected.

To further analyze the regulatory mechanism, we examined mRNAs on which ribosomes assemble either in the cap-stimulated mode or mediated by the IRES from

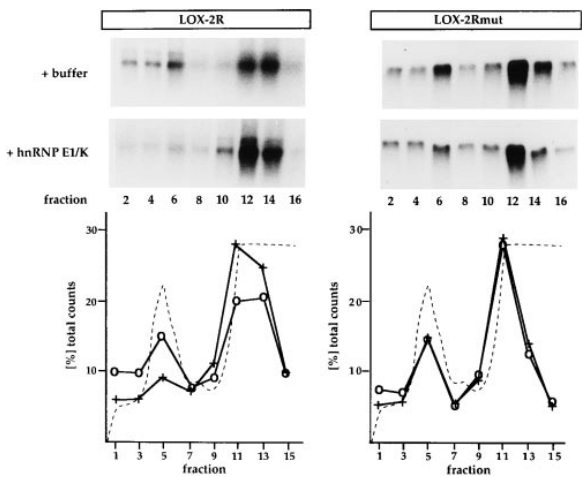


Figure 6. hnRNPs K and E1 Specifically Inhibit 80S Ribosome Assembly

32 P-labeled RNAs were preincubated with dialysis buffer (open circles) or 500 ng of recombinant NTA-agarose purified hnRNP E1/K (1:3 ratio) (plus signs). Translation initiation complexes were subsequently allowed to assemble on the labeled LOX-2R (left panel) or LOX-2Rmut (right panel) mRNAs in cycloheximide-treated rabbit reticulocyte lysate, and resolved by centrifugation in 5%–25% linear sucrose gradients. After fractionation (lower panels), the labeled mRNAs were extracted and analyzed by autoradiography after formaldehyde/agarose gel electrophoresis (even-numbered fractions), or the radioactivity counted in the odd-numbered fractions, expressed as the percentage of total counts recovered, and plotted against the fraction numbers. The dashed line denotes the A₂₅₄ absorption profile, which was identical for gradients with added buffer or recombinant proteins.

encephalomyocarditis virus (Kaminski et al., 1990). LOX-10R mRNA was cotranslated as an internal positive control (Figure 7, upper band in all lanes) in rabbit reticulocyte lysate together with CAT mRNA (Figure 7, lanes 1–4), CAT-10R mRNA (lanes 5–8), IRES-CAT mRNA (lanes 9–12), or IRES-CAT-10R mRNA (lanes 13–16). Due to the construction strategy, both IRES-driven transcripts encode an N-terminally extended CAT fusion protein (compare lanes 9–16 with lanes 1–8). As expected, the translation of LOX-10R (lanes 2, 6, 10, and 14), CAT (lane 2), and CAT-10R (lane 6) mRNAs is diminished by the addition of cap analog, whereas the cap-independent translation of IRES-CAT (lane 10) and IRES-CAT-10R (lane 14) is unaffected. Likewise, recombinant

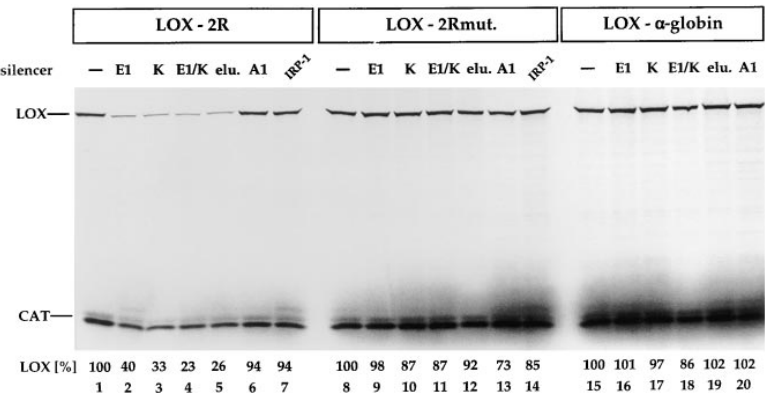


Figure 5. hnRNPs K and E1 Silence LOX mRNA Translation in Wheat Germ Extract LOX mRNAs bearing either two repetitive elements (LOX-2R, lanes 1–7), a mutated version of the two repeat motif (LOX-2Rmut, lanes 8–14), or the α -globin 3' UTR (LOX- α -globin, lanes 15–20) were cotranslated with CAT mRNA (lanes 1–20) as an internal control. Dialysis buffer (lanes 1, 8, and 15); 50 ng of DICE column-purified recombinant proteins hnRNP E1, hnRNP K, hnRNP E1/K (1:3 ratio); DICE column-purified rabbit reticulocyte regulatory proteins; or recombinant proteins hnRNP A1 and IRP-1 were added as indicated. The data were quantitatively evaluated as described in the legend to Figure 2.

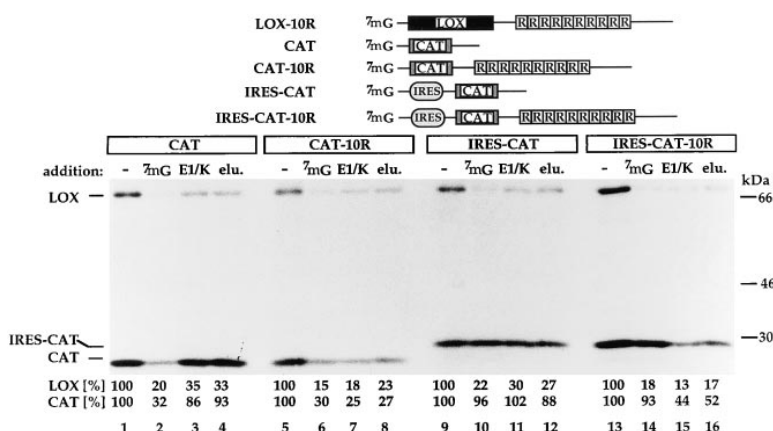


Figure 7. hnRNPs K and E1 Specifically Silence Both Cap-Dependent and IRES-Mediated Translation

LOX-10R mRNA as an internal positive control (lanes 1–16) was cotranslated with the following CAT reporter mRNAs (schematically depicted at the top): CAT (lanes 1–4), CAT-10R (lanes 5–8), IRES-CAT (lanes 9–12), or IRES-CAT-10R (lanes 13–16). Dialysis buffer (lanes 1, 5, 9, and 13), 3.3 mM m⁷GpppG cap-analog (lanes 2, 6, 10, and 14), 500 ng of NTA-agarose-purified hnRNPs E1/K (1:3 ratio, lanes 3, 7, 11, and 15), or 50 ng of DICE-purified rabbit reticulocyte regulatory proteins (lanes 4, 8, 12, and 16) were added to the translation reactions in rabbit reticulocyte lysate. The positions of ³⁵S-methionine-labeled LOX, CAT, and IRES-CAT fusion polypeptides are indicated. Translation of LOX and CAT mRNAs was quantitated by phosphorimaging and expressed as percentages of the signal in lanes without the addition of regulatory proteins.

hnRNPs K and E1 as well as the purified regulatory proteins from rabbit reticulocyte lysate repress LOX-10R (lanes 3, 4, 7, 8, 11, 12, 15, and 16) and CAT-10R (lanes 7 and 8) mRNA translation, without perturbing CAT (lanes 3 and 4) or IRES-CAT (lanes 11 and 12) mRNA. Interestingly, the regulatory proteins also control the translation of IRES-CAT-10R mRNA (lanes 15 and 16), albeit less efficiently. Thus, hnRNPs K and E1 can regulate cap-dependent and IRES-mediated translation initiation, at least in vitro.

Discussion

Translational regulation mediated by 3' UTR elements is a widely used mechanism of posttranscriptional control. We have cloned and expressed two proteins that bind to the DICE located in the 3' UTR of LOX mRNA, and have reconstituted regulation with the recombinant proteins in transfected cells and in vitro. We believe this to represent the first such functional reconstitution of 3' UTR-mediated translational control in eukaryotes. We also provide an initial characterization of the underlying regulatory mechanism.

A Cytoplasmic Function for hnRNP K and hnRNP E1

The data in Figures 2–7 show that the hnRNPs K and E1 function as specific cytoplasmic regulatory proteins of LOX mRNA translation. This finding appears to be surprising, both in terms of the subcellular localization and the broad RNA-binding specificity that is characteristic of hnRNPs. However, previous work on the regulation of alternative splicing in mammalian cells (Mayeda and Krainer, 1992; Min et al., 1995) and the control of dorsoventral axis formation in *Drosophila* (Matunis et al., 1994) have implicated hnRNP A1, hnRNP F, and squid (hnRNPs A/B), respectively, in the control of specific genes. Recent studies have revealed that a distinct subset of hnRNPs, including hnRNP K, shuttle between the nucleus and the cytoplasm, suggesting unexpected functions of these shuttling hnRNPs in nucleocytoplasmic transport and/or in the cytoplasm (Piñol-Roma and

Dreyfuss, 1993). Our findings appear to establish such a cytoplasmic function.

Searching of the EMBL database identified a protein that is closely related to hnRNP E1: hnRNP E2 (Hahm et al., 1993; Leffers et al., 1995; acc. no X78136). We have also expressed this protein, and preliminary analysis suggests that it may regulate LOX translation similar to hnRNP E1 (data not shown). Interestingly, hnRNPs K, E1, and E2 have previously been discussed in the context of gene regulation. The human *c-myc* gene contains a CT-rich promoter element that binds hnRNP K (Takimoto et al., 1993) and mediates hnRNP K-induced transcriptional activation (Michelotti et al., 1996). Furthermore, hnRNPs E1 and E2 have been shown to be components of a ribonucleoprotein complex that binds to a CU-rich region in the 3' UTR of human α -globin mRNA in vitro (α complex) (Kiledjian et al., 1995). The human α -globin mutation Constant Spring is characterized by a reduced stability of the α -globin mRNA and lack of α complex formation (Wang et al., 1995). The suggested involvement of hnRNP E1 in regulating both α -globin mRNA stability and LOX translation via CU-rich 3' UTR-binding motifs prompted us to directly evaluate the α complex-binding region for function as a translational regulatory element. Remarkably, the translation of reporter mRNAs bearing the CU-rich region of α -globin mRNA is not affected by the hnRNPs E1 and K in transfected HeLa cells (Table 1), cell-free translation extracts from wheat germ (Figure 5), or rabbit reticulocytes (data not shown). By contrast, the CU-rich DICE confers translational control under the same conditions. Thus, while the hnRNPs K and E1 show relatively broad specificity for C(U)-rich sequences in biochemical binding assays (Matunis et al., 1992; Kiledjian et al., 1995), they are exquisitely discriminatory in their functional specificity.

Lipoxygenase Regulation during Erythroid Differentiation

LOX mRNA is expressed but translationally silenced in early erythroid cells in the bone marrow and the peripheral blood (Schewe et al., 1986). We have investigated

the association of LOX mRNA with hnRNP K in translationally inactive mRNPs of peripheral blood reticulocytes. The specific association of LOX mRNA and hnRNP K in these mRNPs (Figure 4) supports the role of this protein as a translational silencer of LOX mRNA during erythroid differentiation, although it does not formally prove it.

Our findings raise the question of how erythroid differentiation signals alter the function of hnRNPs K and E1 in LOX mRNA silencing. Several scenarios can be envisaged. The regulatory proteins might be degraded or sequestered; their RNA-binding activity reduced or their RNA-binding specificity might be changed; or their ability to interfere with 80S ribosome assembly could be switched off. The data shown in Figure 3 and Table 1 indicate that the LOX-2R and LUC-2Rmut mRNAs are translated equally well in HeLa cells that express only their endogenous complement of hnRNPs K and E1. Translational silencing in HeLa cells is only observed after cotransfection of additional hnRNPs K and/or E1. A related observation applies to the translation of DICE-containing mRNAs in rabbit reticulocyte lysates (Figure 2). At present, we do not know the level of overexpression or the subcellular localization of these proteins in the transiently transfected cells. The additional copies may titrate a cellular activity that modulates the function of hnRNPs K and E1 as translational silencers. In this respect, it is also interesting that hnRNP K interacts with the SH3 domains of Src (Taylor and Shalloway, 1994; Weng et al., 1994), Fyn, Lyn (Weng et al., 1994), and Vav (Hobert et al., 1994; Bustelo et al., 1995). Vav is a hematopoietic cell-specific, signal-transducing protein that becomes tyrosine phosphorylated after engagement with several cell surface receptors. The hnRNP K/Vav interaction is mediated by two proline-rich sequences in the central region of hnRNP K. Interestingly, p95vav also interacts with a second ~45 kDa poly(rC)-binding protein, which may represent hnRNP E1 and/or E2 (Bustelo et al., 1995), and phosphorylation appears to negatively affect RNA binding by hnRNPs E1 and E2 (Leffers et al., 1995). In addition, hnRNP K binds and is phosphorylated by an IL-1-responsive kinase (Van Seuning et al., 1995). The possible role of these interactions and of the phosphorylation of hnRNPs K and E1 in translational control during erythroid differentiation will warrant further investigation.

Mechanism of Translational Regulation

Binding of regulatory proteins to 3' UTR elements can mediate translational activation or silencing. During oocyte maturation in *Xenopus laevis*, an element upstream from the polyadenylation signal acts as a cytoplasmic polyadenylation element (CPE). CPEB, a 62 kDa protein, appears to activate translation by stimulating poly(A) tail elongation (Hake and Richter, 1994). In *Drosophila*, the anterior pattern determinant bicoid binds to the 3' UTR of caudal mRNA and spatially restricts caudal protein expression by silencing translation (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). In a hybrid mRNA bearing the antennapedia internal ribosome entry site in the intercistronic region between two ORFs and the caudal 3' UTR, bicoid-suppressed translation of the upstream

(cap-dependent), but not the downstream (IRES-mediated), ORF (Dubnau and Struhl, 1996). This result suggests that bicoid may interfere with a cap-dependent step of translation initiation.

By contrast, both cap-dependent and IRES-driven translation are silenced by the LOX regulatory system (Figure 7), suggesting that hnRNPs K and E1 exert their regulatory action at a step downstream of the convergence of the two translation initiation pathways, and excluding eIF4E as the molecular target for the LOX silencing mechanism. Their ubiquitous occurrence and their ability to function in different cell types raises the question of whether hnRNPs K and E1 are also involved in the translational regulation of other mRNAs.

Experimental Procedures

Plasmids

The full-length rabbit reticulocyte 15-LOX cDNA (LOX-10R), the 241 nt fragment with the 3' UTR LOX regulatory region (10R), the nonregulatory 335 nt downstream fragment (NR), and the CAT construct bearing the LOX regulatory region (CAT-10R) have been described (Ostareck-Lederer et al., 1994). The LOX cDNA lacking the 241 nt CU-rich fragment (LOX-NR) was generated by appending the NR fragment 3' to the LOX ORF cloned into pBluescript II-SK (LOX). To generate LOX-2R and LOX-2Rmut, respectively, complementary oligonucleotides representing two repeat elements of the 10R sequence, 5' **AGCTTCCCCA CCTCTTCCCCAAGCCCCA C CCTCTT CCCC AAGAT 3'** (for LOX-2R) and 5' **AGCTTCCCCA AGAGAGACCC CAAGCCCCAA GAGAGACCCC AAGAT 3'** (for LOX-2Rmut, exchanged nucleotides are underlined; flanking sequences for cloning purposes are in bold) were cloned into the HindIII/Clal sites of LOX. LOX mRNA bearing the human α -globin 3' UTR was generated by insertion of a fragment containing nucleotides 1–107 downstream of the α -globin termination codon (Wang et al., 1995; a kind gift of S. Liebhaber) into HindIII/Clal of LOX. IRES-CAT was generated from pEMC2.1 (a kind gift from M. Howell and R. J. Jackson) and contains the IRES of EMCV, followed by the CAT ORF. For the construction of IRES-CAT-10R, the complete 3' UTR of rabbit 15-LOX mRNA was inserted into IRES-CAT downstream of the CAT ORF. For LUC indicator constructs, the luciferase cDNA from pGEM-LUC (Promega) was inserted into pSG5 (Green et al., 1988) to generate LUC. LUC-2R and LUC-2Rmut were made in analogy to LOX-2R and LOX-2Rmut with synthetic oligonucleotides inserted 3' of the ORF of LUC. pSG5-LUC- α -globin was cloned from the α -globin 3' UTR plasmid (Wang et al., 1995) by PCR and insertion 3' to the LUC ORF. pSG5-hnRNP E1 and -hnRNP K were generated by insertion of the cDNAs without the histidine tags. The hGH transfection control vector was pSG5-hGH-TRS3 (Pantopoulos and Hentze, 1995). The hnRNP A1 cDNA was cloned by PCR from a pT7-TT-hnRNP A1 construct (Izaurralde et al., in press) into pSG5. All plasmid constructs were verified by DNA sequencing.

Affinity Purification of LOX Regulatory Proteins

Reticulocytosis in rabbits was induced by venesection on five consecutive days (10–12 ml of blood/kg rabbit per day). The rabbits were exsanguinated two days later by cardiac puncture. Reticulocyte extracts (S100) (Thiele et al., 1982) and the biotinylated RNA transcripts 10R and NR were prepared exactly as described (Ostareck-Lederer et al., 1994). All subsequent procedures were performed at 4°C. Aliquots (1 ml) of S100 extract were preincubated for 15 min with 100 U RNAGuard (Pharmacia) in the presence of 2% 2-mercaptoethanol, and the supernatant was collected after 1 min centrifugation at 3000 rpm in an Eppendorf centrifuge. Packed streptavidine-agarose beads (300 μ l; GIBCO-BRL) were washed four times with purification buffer (150 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl [pH 7.4], 0.5 mM DTT), and incubated with 10R or NR transcripts (~2 μ g) for 1 hr. The S100 extract was tumbled with the transcript/beads affinity resin for 100 min, followed by four washes (10 min each) with 1.0 ml of purification buffer. Proteins bound to the affinity resin were collected in three consecutive elutions (10 min each) with 150 μ l of buffer (3 M KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl [pH 7.4], 0.5

mM DTT) and pooled, followed by dialysis against multiple changes of 10 mM CH_3COOK , 0.3 mM MgCl_2 , 10 mM Tris-HCl (pH 7.4), 1 mM DTT for 3 hr. Aliquots (10 μl) of the wash and elution fractions were analyzed by SDS-PAGE and silver staining.

Peptide Purification and Sequencing

Protein bands were excised from silver-stained gels and digested in-gel as described (Shevchenko et al., 1996; Wilm et al., 1996a). For nanoelectrospray tandem mass spectrometry (MS) analysis, the peptide solution was desalted on a 50 nl microcartridge (Wilm and Mann, 1996) of Poros R2 material (Perseptive Biosystems, Framingham, MA). A glass capillary with the unseparated peptide mixture was mounted in the nanoelectrospray ion source for mass spectrometric analysis on a tandem mass spectrometer API III (PE-Sciex, Ontario, Canada) equipped with a nanoelectrospray ion source (Wilm and Mann, 1994, 1996). Peptide mass spectra (Q1 scans) were recorded with 0.1 Da step width and unit mass resolution. Parent ion scans for the immonium ion of isoleucine and leucine to specifically detect isoleucine/leucine-containing peptides were performed with 0.2 Da step width and a lower resolution (2 Da peak width at 50% height) to increase sensitivity (Wilm et al., 1996b). For the tandem MS investigation, Q1 was set to transmit a 2 Da mass window. The tandem MS spectra were acquired with 0.2 Da step width. The mass resolution was set such that identifiable Y'' ions (Roepstorff and Fohlman, 1984) could be assigned to better than 0.5 Da. Peptide sequence tags (Mann and Wilm, 1994) were assembled using Apple Scripts, and a nonredundant database comprising more than 200,000 sequences was searched. No restrictions on molecular weight, pI (isoelectric point), or species of origin were applied. The retrieved sequence was verified by comparing it to the entire fragment spectrum using Bio-MultiView (PE-Sciex).

The following peptides were identified from two independent analyses: five peptides for the 66 kDa protein, corresponding to hnRNP K: IITITGTQDQIQNAQYLLQNAQYLLQNSVK, LLHQSLAGGIIVK, IILDLSIESPIKGR, NLPLPPPPPPR, and ILSISADIETIGEL; six fragments for the 48 kDa band, corresponding to the C-terminal region of hnRNP K: IITITGTQDQIQNAQYLLQNSVK, GSYGDLGGPIITQVTIPK, DLAGSIIGKGGQRIK, LLHQSLAGGIIVK, IILDLSIESPIKGR, and NLPLPPPPPPR; and one peptide that is uniquely found in hnRNP E1: IITLTGPTNAIFK.

Expression of Recombinant Proteins

The cDNAs for hnRNP E1 and hnRNP K were PCR amplified from a HeLa cell cDNA library (gift of H. Stunnenberg). Gel-purified PCR-products were cloned into pET16b (Novagene) for transformation of *E. coli* BL21 (DE3). Transformed bacteria were grown in LB medium at 37°C to a density of 0.6–0.8 OD_{600} , pelleted, washed with minimal medium, and induced for 4 hr in minimal medium with 10 mM isopropyl-1-thio- β -D-galactopyranoside at 23°C. The cells were lysed (20 mM Tris-HCl [pH 8.0], 250 mM NaCl, 0.25% NP-40) by freeze thawing and subsequent sonication for 1 min with a Branson Sonifier B15 in the presence of 0.1% PMSF and 10 $\mu\text{g}/\text{ml}$ leupeptin. After centrifugation for 10 min at 6000 rpm in a Sorvall SS34 rotor, the His-tagged proteins were purified from the supernatant on Ni^{2+} -NTA-Agarose beads (Quiagen). The eluate (200 mM imidazol for hnRNP E1, 500 mM imidazol for hnRNP K) was dialyzed extensively against the buffer used for the affinity-purified proteins. For purification on a DICE column, the procedure described for rabbit reticulocyte lysate was followed. The final recovery was ~10% (hnRNP E1) and ~2% (hnRNP K). Recombinant IRP-1 (Gray et al., 1993) and hnRNP A1 (Izaurralde et al., in press) were prepared as described.

In Vitro Transcription

Uncapped ^{32}P -labeled RNAs (specific activity 7×10^7 cpm/ μg) or unlabeled competitor RNAs for UV cross-linking assays were transcribed with T3 RNA polymerase (Stratagene) and purified as described (Ostareck-Lederer et al., 1994). Capped mRNAs (>95% capping efficiency) were generated as described (Gray et al., 1993); excess cap analog was removed after transcription and phenol/chloroform extraction by two rounds of chromaspin-100 (Clontech). For translation initiation assays, capped ^{32}P -labeled mRNAs were transcribed and purified as above (specific activity 5×10^7 cpm/ μg).

UV Cross-Linking Assays

UV cross-linking assays were carried out exactly as described (Ostareck-Lederer et al., 1994), using as input material S100 lysate (120–150 μg of protein per μl) from rabbit reticulocytes or the indicated amounts of purified rabbit or recombinant human proteins.

Cell-Free Translations

The capped, nonpolyadenylated mRNAs were preincubated with the regulatory proteins or appropriate controls for 10 min on ice. Standard 12 μl reactions contained 8 μl of RRL (Jackson and Hunt, 1983) adjusted to 100 mM CH_3COOK , 0.5 mM MgCl_2 , and 0.1 mM amino acids without methionine; 0.5 U of InhibitAce (5Prime–3Prime); 1.0 μl of capped mRNAs (10 ng of LOX [10R] or LOX [NR], 1 ng of CAT); and 0.5 mCi/ml [^{35}S] Met (Amersham, SJ1015). In Figure 7, a 12 μl reaction contained 4.8 μl of RRL (Promega), and the final concentration of the components added was 10 mM CH_3COOK , 0.3 mM MgCl_2 , and 0.06 mM amino acids without methionine; 0.5 U of InhibitAce; 10 ng of poly(C); 1 μl of capped mRNAs; and 0.7 μl of [^{35}S] Met (Amersham, SJ1015, 10 mCi/ml). The addition of 10 ng of poly(C) was dispensable when 8 μl (instead of 4.8 μl) of RRL was used. Where indicated, RRL was preincubated with 3.3 mM m⁷GpppG cap analog for 3 min at 30°C. Translation reactions were incubated at 30°C for 1 hr and stopped by incubation with RNase A (Jackson and Hunt, 1983). Translation in micrococcal nuclease-treated wheat germ extract (WGE) (Promega) was performed according to the manufacturer's protocol using 12.5 ng of capped LOX-2R, LOX-2Rmut, or LOX- α -globin and 1 ng of capped CAT mRNAs.

Translation Initiation Assays and Sucrose

Gradient Analysis

RRL was preincubated with 0.5 mM cycloheximide for 3 min at 30°C. Labeled LOX-2R or LOX-2Rmut mRNAs (4 ng) was incubated for 10 min at 4°C with recombinant NTA-agarose-purified hnRNPs E1 (125 ng) and K (375 ng), or with dialysis buffer. Initiation reactions at 30°C were stopped after 5 min by addition of ice-cold dilution buffer, and initiation complexes resolved on linear 5%–25% sucrose gradients (Gray and Hentze, 1994). Sixteen fractions (250 μl each) were collected from the bottom of the gradient and were analyzed by scintillation counting or RNA extraction and electrophoresis through formaldehyde agarose gels.

In Vivo Analyses

HeLa cells were transiently transfected by the calcium phosphate method of Graham and van der Eb (1973) with 5 μg of LUC-2R, LUC-2Rmut, or LUC- α -globin, 5 μg of hGH control plasmid, plus 5 or 10 μg of pSG5-hnRNP E1 or pSG5-hnRNP K, alone or in combination, 10 μg of pSG5 IRP-1 (Kollmus et al., 1996), or 10 μg of pSG5 hnRNP A1. The quantity of expression plasmids for RNA-binding proteins was equalized by addition of pSG5-U1A (Stripecke et al., 1994) where necessary. Analysis by metabolic labeling and immunoprecipitation was performed as described (Stripecke et al., 1994) using saturating quantities of polyclonal anti-hGH (Daco), anti-luciferase (a gift of H. Hauser), and anti-ferritin (Boehringer Mannheim) antibodies. Luciferase assays were carried out according to Brasier et al. (1989). Total cellular RNA was isolated using the RNA-Clean System (AGS), and Northern blots were probed with a ^{32}P -dCTP-labeled luciferase cDNA.

To analyze the association of LOX mRNA with hnRNP K in reticulocyte RNP, S100 lysate was prepared and mRNPs pelleted after removal of polysomes at $300000 \times g$ for 180 min. The RNPs were resuspended in TKM (50 mM Tris [pH 7.6], 50 mM KCl, 5 mM Mg (CH_3COO)₂) to a protein concentration of 15 $\mu\text{g}/\mu\text{l}$. RNPs (100 μl) were incubated with 1 μl of preimmune serum, hnRNP K polyclonal antibody (a kind gift of K. Bomsztyk), or water for 1.5 hr at 4°C, followed by an addition of 50 μl of protein A-Sepharose (Pharmacia) for 1.5 hr. The beads were washed three times with buffer (10 mM HEPES [pH 7.2], 3 mM MgCl_2 , 1 mM DTT), and the pellets resuspended in 200 μl of PK buffer (100 mM Tris [pH 7.5], 12.5 mM EDTA, 150 mM NaCl, 1% SDS) with 4 μl of proteinase K 20 mg/ml (Sigma) and 20 μg of glycogen (Boehringer Mannheim). The supernatant (100 μl) received 100 μl of 2 $\times$ PK buffer, proteinase K, and glycogen. The samples were incubated at 50°C for 45 min, and pellets removed

by centrifugation, extracted twice with phenol/chloroform, and ethanol precipitated. The recovered RNAs were analyzed by Northern blotting.

Acknowledgments

Correspondence regarding this paper should be addressed to M. W. H. (Hentze@EMBL-Heidelberg.de). We thank Fatima Gebauer, Thomas Preiss, and Iain Mattaj for constructive comments on the manuscript; Richard Jackson, Steve Liebhaber, and Iain Mattaj for plasmids; Nina Vagner for recombinant hnRNP A1 protein; Henk Stunnenberg for the HeLa cell cDNA library; Hansjoerg Hauser for the luciferase antibodies; and Karol Bomsztyk for the hnRNP K antibodies. A. O.-L. was supported by fellowships from Boehringer Ingelheim and EMBO. M. W. H. acknowledges support from the Deutsche Forschungsgemeinschaft (He 1442/2-3) and the European Commission Biotechnology Program (BIO4-CT95-0045).

Received August 19, 1996; revised February 28, 1997.

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