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Lipoxygenase mRNA Silencing in Erythroid Differentiation: The 3′UTR Regulatory Complex Controls 60S Ribosomal Subunit Joining

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Summary

15-lipoxygenase (LOX) expression is translationally silenced in early erythroid precursor cells by a specific mRNA–protein complex formed between the differentiation control element in the 3′ untranslated region (UTR) and hnRNPs K and E1. The 3′UTR regulatory complex prevents translation initiation by an unknown mechanism. We demonstrate that the 40S ribosomal subunit can be recruited and scan to the translation initiation codon even when the silencing complex is bound to the 3′UTR. However, the joining of the 60S ribosomal subunit at the AUG codon to form a translation competent 80S ribosome is inhibited, unless initiation is mediated by the IGR-IRES of the cricket paralysis virus. These findings identify the critical step at which LOX mRNA translation is controlled and reveal that 60S subunit joining can be specifically regulated.

Introduction

Translational control governs important decisions during somatic and germ cell differentiation as well as in embryonic development (Curtis et al., 1995; Stebbins-Boaz and Richter, 1997; Wickens et al., 2000). Regulation of translation can be achieved by modulation of the activity of general translation initiation factors, or by specific interactions between control sequences located in the 5′ and/or 3′ untranslated regions (UTR) of mRNAs and regulatory proteins. The 3′UTR has emerged as a particularly common site for such regulatory interactions, with abundant examples from invertebrates to mammals (Sallés et al., 1994; Gavis et al., 1996; Ha et al., 1996; Wormington et al., 1996; Goodwin et al., 1997; Jan et al., 1997; Zhang et al., 1997; Wharton et al., 1998; Stebbins-Boaz et al., 1999; Thompson et al., 2000; Wickens et al., 2000).

Ribosome assembly on cellular mRNAs begins with the recruitment of the small ribosomal subunit in the form of a 43S translation preinitiation complex (reviewed in Sachs et al., 1997; Gingras et al., 1999). The 5′ m^7GpppN cap structure and its bound initiation factor (eIF)4F direct the 43S complex to the 5′ end of the mRNA. Subsequently, the 5′UTR is "scanned" to identify the translation initiation codon to which the 43S complex repositions (Kozak, 1989). While these steps do not require GTP hydrolysis, the final joining of the large ribosomal subunit to form a stable, translation-competent 80S ribosome necessitates GTP hydrolysis on both eIF2-GTP and eIF5B-GTP (Merrick and Hershey, 1996; Pestova et al., 2000).

The recruitment of the small ribosomal subunit usually represents the rate limiting step in translation initiation (Jackson, 1996; Mathews et al., 1996). It is thus not surprising that translational control usually targets this early step. Examples of regulated 43S recruitment include the 4E-BPs, small growth signal-regulated proteins which interact with the cap binding protein eIF4E (Pause et al., 1994; Gingras et al., 1999) as well as the heat shock-induced Hsp27, which sequesters eIF4G (Cuesta et al., 2000). Both mechanisms block 43S recruitment by inhibiting eIF4F formation. Different viruses have developed strategies to interfere with 43S complex binding by cellular mRNAs at the level of eIF4G (Schneider, 1995; Ehrenfeld, 1996). Binding of IRP-1 to the IRE in the 5′UTR of ferritin mRNA prevents the recruitment of the 43S complex to prebound eIF4F (Gray and Hentze, 1994; Muckenthaler et al., 1998). Recently, the protein Maskin was suggested to regulate translation during *Xenopus* oocyte maturation by interfering with the eIF4E/eIF4G interaction and thus blocking the translation of mRNAs with CPEs in their 3′UTRs (Stebbins-Boaz et al., 1999). With the notable exception of the translational regulation of GCN4 expression in yeast, where the frequency at which initiation-competent 43S complexes reach the initiation codon is regulated (Hinnebusch, 1996, 1997), it remains an open question whether the translation initiation pathway is regulated after recruitment of the 43S translation preinitiation complex has occurred. Furthermore, the common usage of 3′UTR-mediated translational control raises the question of how ribosome assembly at the 5′ terminus of the mRNA is regulated from the opposite end.

Reticulocyte 15-lipoxygenase (LOX) mRNA is an excellent example of translational control from the 3′UTR (Hunt, 1989). The LOX protein is only expressed in erythroid cells just before they become mature erythrocytes and mediates mitochondrial breakdown (Rapoport and Schewe 1986; van Leyen et al., 1998). This temporal restriction is achieved by translational silencing of the mRNA in erythroid precursor cells (Höhne et al., 1988). The 3′UTR differentiation control element (DICE) binds the KH-domain proteins hnRNP K and E1, which leads to the establishment of a translationally silenced mRNP in erythroid precursor cells and in transfected cells (Ostareck-Lederer et al., 1994; Ostareck et al., 1997). Using recombinant hnRNP K and E1 as well as a fully functional DICE of 38 nucleotides (fDICE), we reconstituted LOX mRNA silencing in a cell-free translation extract from rabbit reticulocytes (Ostareck et al., 1997). We showed that silencing occurs at the level of translation initiation, and that it can control cap-dependent translation as

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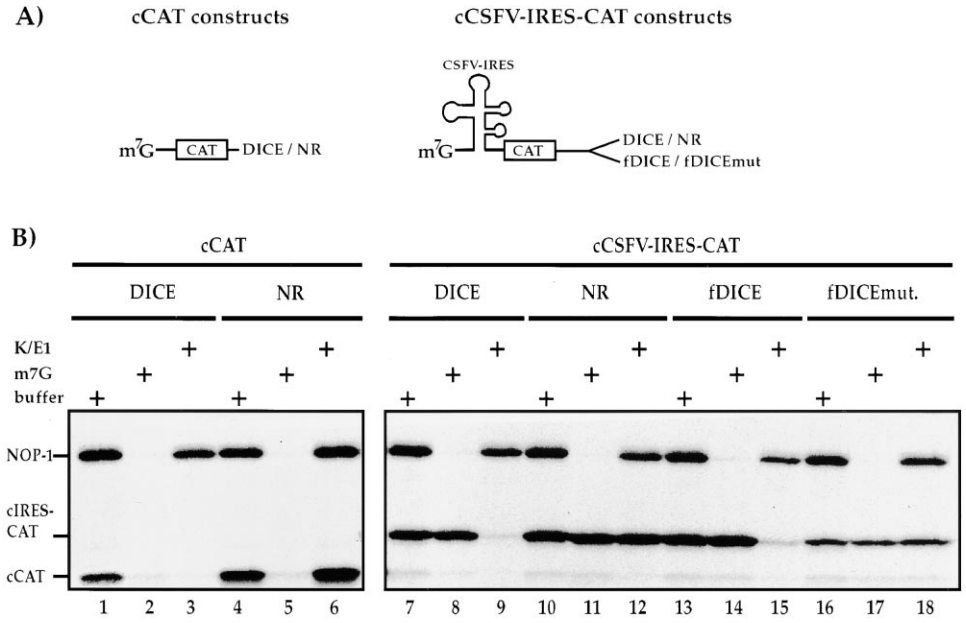

Figure 1. HnRNPs K and E1 Specifically Silence Translation Driven by the CSFV-IRES

(A) Schematic drawing of CAT and CSFV-IRES-CAT reporter mRNAs carrying different 3′UTR sequences used in in vitro translation.
(B) NOP-1 mRNA as an internal control (lanes 1–18) was cotranslated with the following CAT-reporter mRNAs: CAT-DICE (lanes 1–3) and CAT-NR (lanes 4–6) and the CSFV-IRES containing reporter mRNAs: CAT-DICE (lanes 7–9), CAT-NR (lanes 10–12), CAT-fDICE (lanes 13–15), and CAT-fDICEmut (lanes 16–18). Dialysis buffer (lanes 1, 4, 7, 10, 13, and 16), 0.5 mM m^7GpppG cap-analog (lanes 2, 5, 8, 11, 14, and 17) or hnRNPs K and E1 (lanes 3, 6, 9, 12, 15, and 18) were added to the translation reactions in rabbit reticulocyte lysate. The positions of ^{35}S-Met-labeled CAT, CSFV-IRES-CAT, and NOP-1 peptides are indicated on the left.

well as translation mediated by the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) (Ostareck et al., 1997). Here, we address the question of how this is achieved. Surprisingly, we find that the final step in the translation initiation pathway is targeted by the 3′UTR complex. Translation initiation via the IGR-IRES of the cricket paralysis virus, which solely requires 40S and 60S ribosomal subunits without any eIFs (Wilson et al., 2000a), is shown to bypass the silencing mechanism. This implicates the eIFs involved in 60S joining as the possible molecular targets for the hnRNP K/E1 complex.

Results

The hnRNP K/E1-DICE Complex Silences eIF4F-Independent Translation

Our earlier results that the inhibitory complex silences EMCV IRES-dependent translation excluded the cap binding protein eIF4E as the primary regulatory target (Ostareck et al., 1997), but were fully consistent with the possibility that it controlled the recruitment of the small ribosomal subunit to LOX mRNA. If this were the case, the IRES of the Classical Swine Fever Virus (CSFV) may bypass the regulatory block, because initiation on the CSFV-IRES entails an entirely different mechanism of 40S recruitment and does not require eIF4E, -4G, -4A, or eIF4B, nor eIF1 or eIF1A (Pestova et al., 1998).

A set of reporter mRNAs bearing a 5′ m^7GpppG cap (Figure 1A) was translated in micrococcal nuclease–treated rabbit reticulocyte lysate (RRL) together with NOP-1 mRNA as an internal specificity control (Figure 1B, upper band in all lanes). CAT-DICE (Figure 1B, lanes 1–3) and CAT-NR (Figure 1B, lanes 4–6) are translated by a cap-dependent mechanism and serve as positive and negative controls, respectively, for the specificity of translational silencing mediated by the addition of recombinant hnRNPs K/E1. Two sets of CSFV-IRES containing mRNAs were tested: one set carries the full-length DICE from rabbit 15-LOX mRNA (CAT-DICE; Figure 1B, lanes 7–9) or the 15-LOX 3′UTR with a deletion of the DICE (CAT-NR; Figure 1B, lanes 10–12); the other set harbors the 38 nucleotide synthetic DICE (CAT-fDICE; Figure 1B, lanes 13–15) or a mutated version thereof (CAT-fDICEmut; Figure 1B, lanes 16–18). Due to the construction strategy, the CSFV-IRES-driven mRNAs encode an N terminally extended CAT fusion protein (compare lanes 1–6 with lanes 7–18).

As expected, the translation of NOP-1 (lanes 2, 5, 8, 11, 14, and 17), CAT-DICE (lane 2) and CAT-NR (lane 5) mRNAs is fully inhibited by the addition of m^7GpppG cap analog, demonstrating that these mRNAs are translated by a cap-dependent mechanism. By contrast, the translation of the CSFV-IRES-CAT mRNAs (lanes 8, 11, 14, and 17) is unaffected by this treatment, showing that these mRNAs are translated via the IRES. Importantly, recombinant hnRNPs K and E1 repress the translation of CSFV-IRES-CAT-DICE (lane 9) and CSFV-IRES-CAT-fDICE (lane 15) mRNAs, without perturbing the translation of CSFV-IRES-CAT-NR (lane 12), CSFV-IRES-CAT-fDICEmut (lane 18), NOP-1 (lanes 3, 9, and 15), or CAT-NR (lane 6) mRNAs. We conclude that the silencing mechanism is also effective against CSFV-IRES-mediated translation. This suggests the possibility that the

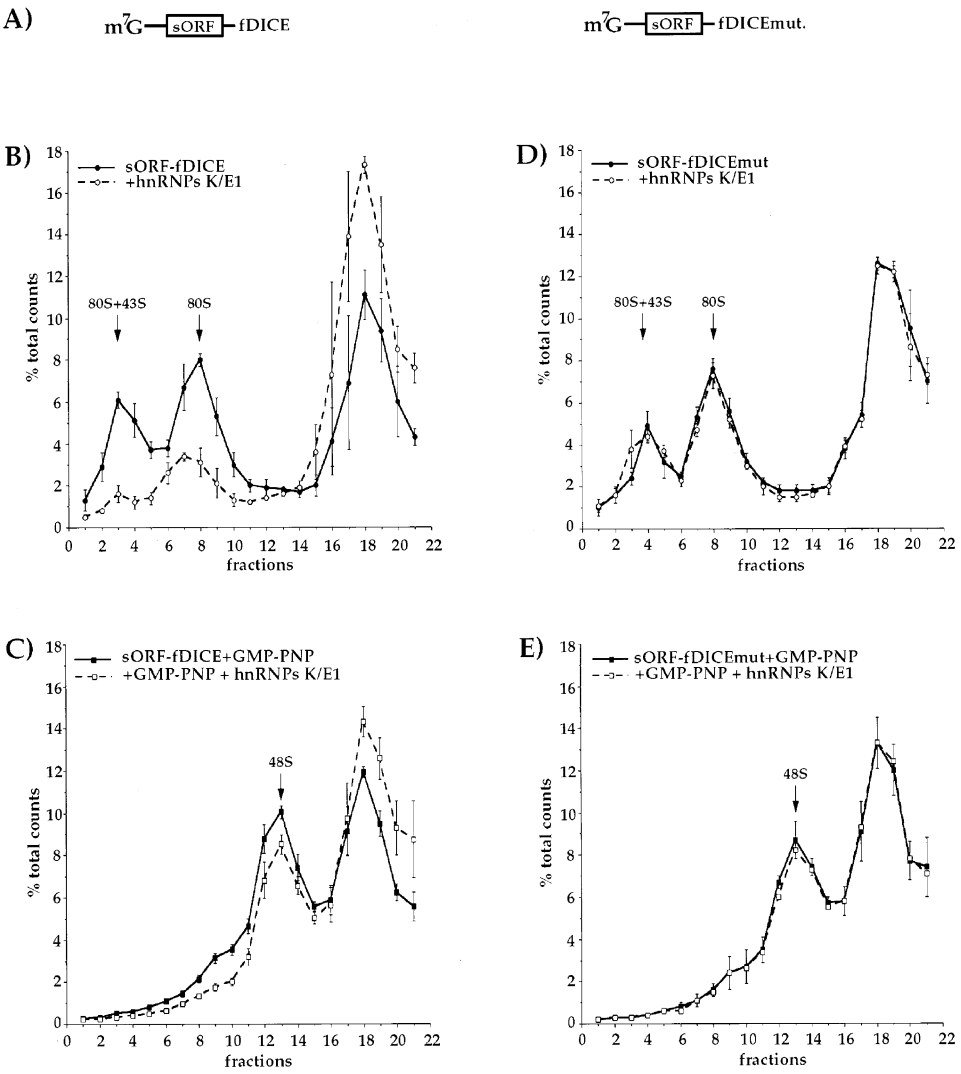

Figure 2. HnRNPs K and E1 Inhibit 80S Ribosome Assembly but Do Not Affect 48S Complex Formation in Cap-Dependent Translation

(A) Schematic representation of the mRNAs employed in in vitro translation initiation reactions, sORF-fDICE and sORF-fDICEmut. (B) ^{32}P-labeled sORF-fDICE mRNA was preincubated either with dialysis buffer (filled circles) or hnRNPs K and E1 (open circles), and (C) with 2 mM GMP-PNP (filled squares) or GMP-PNP + hnRNPs K/E1 (open squares). (D) ^{32}P-labeled sORF-fDICEmut mRNA was preincubated either with dialysis buffer (filled circles) or hnRNPs K/E1 (open circles), and (E) with 2 mM GMP-PNP (filled squares) or GMP-PNP + hnRNPs K/E1 (open squares). Translation initiation complexes were subsequently allowed to assemble on the mRNAs in cycloheximide-treated rabbit reticulocyte lysate, and resolved by centrifugation in 5%–25% linear sucrose gradients. After fractionation from the bottom to the top of the gradient, the radioactivity was monitored, expressed as the percentage of total counts recovered, and plotted against the fraction number.

silencing mechanism may act on a step of the translation initiation pathway that is shared by cap-dependent and CSFV-IRES-driven translation, possibly after the small ribosomal subunit has been recruited to the mRNA.

Silencing Acts Downstream of Small Ribosomal Subunit Recruitment

To directly assess initiation complex formation on a DICE-regulated mRNA, we used sucrose gradient analysis of initiation complexes assembled during a 5 min incubation in rabbit reticulocyte lysate. Previously, we had shown that addition of recombinant hnRNPs K and E1 inhibited 80S ribosomal complex formation (Ostareck et al., 1997). However, binding of the small ribosomal subunit (48S complex formation) could not be reliably assessed on mRNAs of over 2 kb length under these conditions (Gray and Hentze, 1994; data not shown), so that it was necessary to design shorter indicator mRNAs to examine the effect of regulation by a 3′UTR DICE. Figure 2A schematically depicts sORF-fDICE and sORF-fDICEmut mRNAs, both of which are only 183 nucleotides long and efficiently form 80S ribosome-associated complexes (Figures 2B and 2D, filled circles, fractions 1–10). They specifically respond to the addition of recombinant hnRNPs K and E1 by inhibition of 80S complex formation (open circles), confirming our earlier findings with LOX-2R mRNA (Ostareck et al., 1997). Addition of the nonhydrolyzable GTP analog GMP-PNP to initiation reactions stalls 43S ribosomal preinitiation complexes at the initiator AUG (Hershey and Monro, 1966;

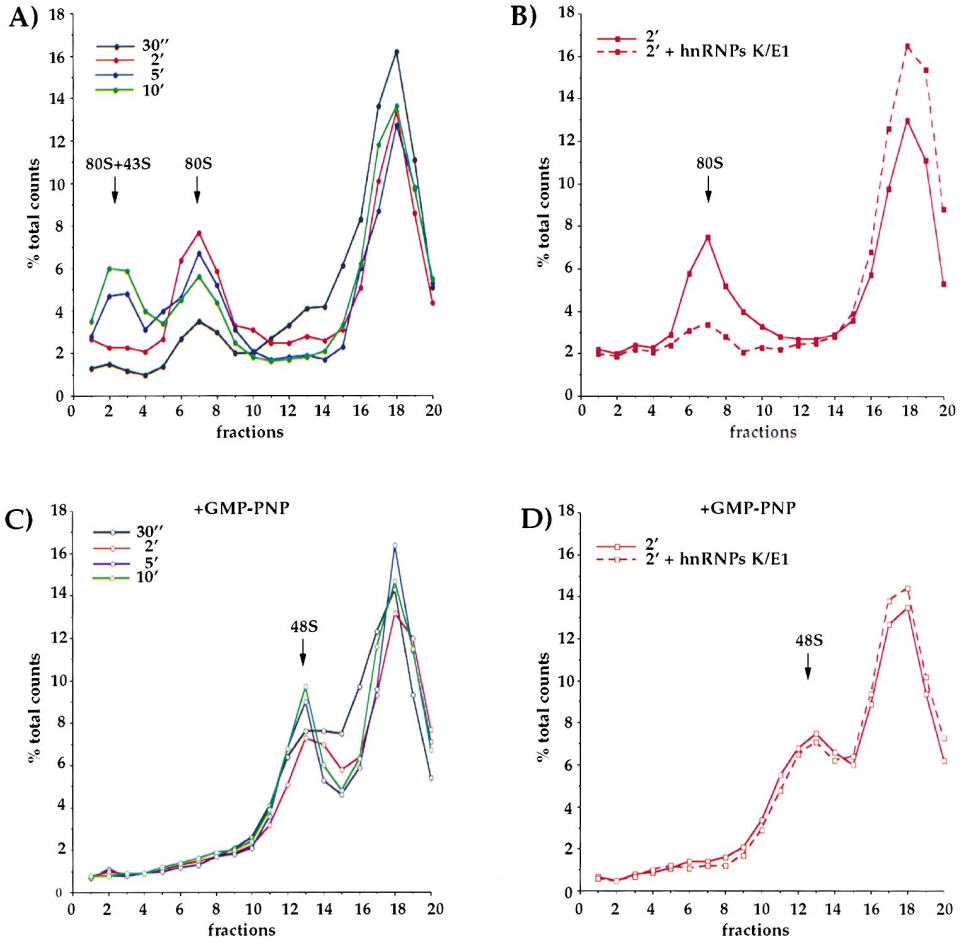

Figure 3. The DICE Silencing Complex Does Not Kinetically Delay 43S Preinitiation Complex Binding

(A) ^{32}P-labeled sORF-fDICE mRNA was incubated in cycloheximide-treated rabbit reticulocyte lysate for 30 s, 2, 5, or 10min, and (B) for 2 min in the absence (continuous line) or presence (dashed line) of hnRNPs K/E1. (C) ^{32}P-labeled sORF-fDICE mRNA was translated as described in (A) in the presence of 2 mM GMP-PNP for 30 s, 2, 5, or 10 min, and (D) for 2 min in the absence (continuous line) or presence (dashed line) of hnRNPs K/E1. Translation initiation complexes were allowed to assemble, and subsequently resolved by sucrose gradient fractionation as described in Figure 2. Radioactivity was monitored and the data are expressed as described above.

Anthony and Merrick, 1992), reflecting the necessity of GTP hydrolysis for 60S subunit joining. Such initiation complexes stalled on sORF-fDICE and sORF-fDICEmut mRNAs, respectively, can be clearly resolved when GMP-PNP is added to the initiation reactions (Figures 2C and 2E, filled squares, fractions 11–15). In contrast to the effect on 80S complex formation, addition of hnRNPs K and E1 has very little effect on the formation of these 48S complexes (open squares).

To address whether the silencing complex may kinetically delay 48S complex formation, which could be masked in a translation complex assembly reaction of 5 min duration, time course experiments were performed with the sORF-fDICE mRNA. As shown in Figure 3A, 80S complexes can already be discerned after 30 s of incubation. 80S complex formation is increased after 2 min. After 5 and 10 min of incubation, faster sedimenting complexes form at the expense of the 80S complexes, which likely represent mRNAs with an 80S ribosome arrested at the AUG codon and a 43S complex "waiting" on the 5′UTR. When the same kinetic experiment is repeated in the presence of GMP-PNP, 48S complexes continue to accumulate until 5 min (Figure 3C). This strongly argues against the possibility of a masked kinetic delay as an explanation for the result shown in Figure 2C. To formally exclude this possibility, the effect of hnRNPs K and E1 on 48S complex formation was monitored in a 2 min reaction. As evident from Figure 3D, 48S complex formation is not affected during this shorter incubation time under conditions where 80S complex formation is strongly inhibited (compare D and B). We conclude that the small ribosomal subunit is recruited to the silenced mRNA, consistent with the findings with the CSFV-IRES. By implication, the joining of the 60S ribosomal subunit to form 80S ribosomes appears to be inhibited or, alternatively, the small ribosomal subunit may bind to the 5′ end of the mRNA but fail to reach the AUG codon in the presence of the 3′UTR silencing complex.

Interestingly, we noticed a difference regarding 48S complex formation on sORF-fDICE mRNA in the presence of hnRNPs K and E1 depending on whether GTP

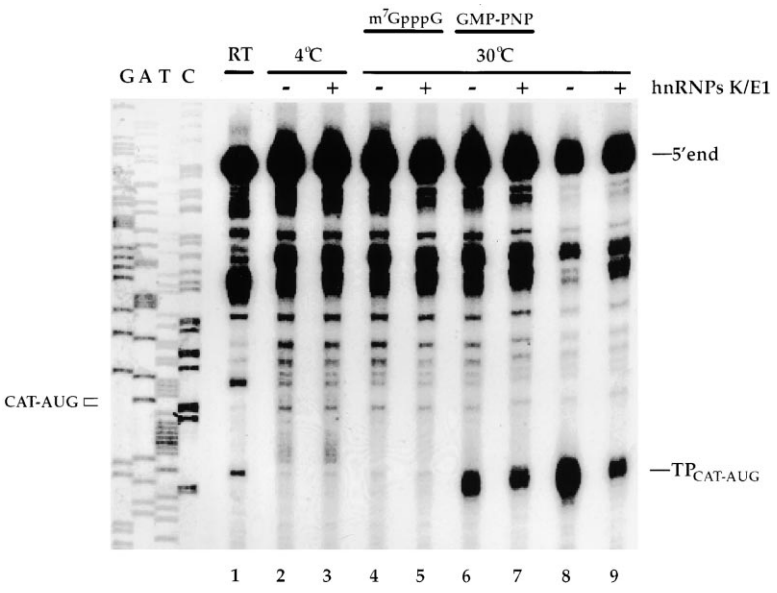

Figure 4. Toe Printing of Ribosomal Complexes Assembled on a Cap-Dependent mRNA Template in Rabbit Reticulocyte Lysate

The products of the AMV reverse transcriptase control reaction on sCAT-fDICE mRNA from primer RT-2 are shown in lane 1. Initiation complex assembly was allowed on the same mRNA for 5 min at 4°C (lanes 2 and 3) or 30°C (lanes 4–9) in rabbit reticulocyte lysate in the presence of 0.5 mM cycloheximide. m^7GpppG (lanes 4 and 5) or GMP-PNP (lanes 6 and 7) were added to the reaction as inhibitors of initiation complex formation before the addition of mRNA. hnRNPs E1 and K were added in lanes 3, 5, 7, and 9. Toe prints resulting from the stop of the reverse transcriptase reaction using primer RT-2 appear 15–17 nt 3′ from the initiator AUG of sCAT-fDICE ($TP_{CAT-AUG}$, lanes 6–9). Lanes G, A, T, and C represent the negative strand sequence of sCAT-fDICE, synthesized from RT-2.

hydrolysis is permitted or not: when GTP hydrolysis is allowed, the inhibition of 80S formation does not result in an accumulation of 48S complexes. Instead, the mRNA repartitions to the top of the gradient (Figure 2B, open circles; Figure 3B, dashed line), indicating that the association of the small ribosomal subunit is not stable under these conditions. Since the presence or absence of GMP-PNP should not differentially affect translation initiation before the AUG codon is reached by the 43S complex, this observation supports the interpretation that the AUG codon is reached by the 43S complex but 60S joining inhibited when the hnRNP K/E1-DICE complex inhibits translation.

Ribosomal Subunits "Toe Print" at the AUG Codon of a Silenced mRNA

We then wanted to directly examine the exact position that the 43S complex reaches on a silenced mRNA. The position of 43S or 80S ribosomal complexes on an mRNA can be identified by stops of reverse transcriptase extension reactions from a primer that anneals to the open reading frame ("toe printing"; Pestova et al., 1996; Wilson et al., 2000a). As shown in Figure 4, a correctly positioned toe print is observed approximately 15–17 nucleotides downstream from the translation initiation codon when 80S complex formation is permitted but translation elongation blocked by cycloheximide (lane 8). This toe print is specific and cap dependent, because it is lacking from a reverse transcription reaction with naked mRNA (lane 1) or with mRNA which was incubated in the rabbit reticulocyte lysate under conditions that do not allow cap-dependent initiation (i.e., incubation at 4°C in lanes 2 and 3, addition of cap analog in lanes 4 and 5). Notably, the toe print near the initiation codon is the only reverse transcription arrest that is specifically seen under conditions that allow 80S (lane 8) or 48S (lane 6) complex formation on the mRNA. Under silencing conditions in the presence of hnRNPs K and E1, the specific toe prints at the initiation codon still appear (lanes 7 and 9). In full agreement with the analysis of initiation complexes on sucrose gradients (Figures 2C and 3D), there is only a minor quantitative effect of the silencing complex on toe print formation under conditions where GTP hydrolysis is blocked with GMP-PNP (compare lanes 6 and 7). As was seen in Figures 2B and 3B, the 43S translation preinitiation complex at the AUG codon appears to be less stable when the mRNA is silenced in the absence of GMP-PNP (compare lanes 8 and 9). We conclude that translation initiation on a silenced mRNA proceeds through 43S complex recruitment and "scanning".

The Silencing Mechanism

Finally, we wanted to address how the 3′UTR regulatory complex inhibits 60S ribosomal subunit joining. We first investigated whether hnRNPs K and E1 can bind to the 40S or 60S ribosomal subunits. Experiments in rabbit reticulocyte lysate using recombinant hnRNPs K and E1 in a DICE-bound or RNA-free state did not yield any evidence for a specific association of the hnRNPs with either of the two ribosomal subunits (data not shown). We reasoned that two principal silencing mechanisms needed to be considered: first, that the hnRNP K/E1 silencing complex (which may or may not include additional factors) inhibits the function of one (or more) of the translation initiation factors that mediate 60S ribosomal subunit joining after the 43S complex has reached the translation initiation codon; second, that the silencing complex may occlude critical surfaces for subunit joining on either (or both) of the ribosomal subunits. To address these possibilities, we constructed CrPV-IGR-IRES-CAT-fDICE and CrPV-IRES-CAT-fDICEmut (Figure 5A). The translation of these mRNAs is driven by the IGR-IRES of cricket paralysis virus (CrPV) via a recently discovered, unusual translation initiation mechanism that involves direct 80S ribosome formation on the translation initiation codon without requirement for any eIFs. It is currently not clear whether preformed 80S complexes can be recruited directly to the IGR-IRES or whether 40S and 60S subunits bind successively (Wilson

et al., 2000a). If the silencing mechanism acted through any of the eIFs involved in ribosomal subunit joining, CrPV-IGR-IRES-mediated translation should not be silenced, because ribosome formation occurs independent of them. If, however, the silencing complex occluded critical sites on either of the two ribosomal subunits, it could be active on CrPV-IGR-IRES-CAT-fDICE mRNA. As shown in Figure 5B, CrPV-IGR-IRES-CAT-fDICE mRNA translation clearly bypasses the silencing mechanism (compare lanes 1 and 2). Importantly, LOX mRNA, which was included as an internal positive control, is silenced.

These findings implicate the eIFs involved in 60S joining as likely molecular targets for the hnRNP K/E1 complex. However, since the CrPV-IGR-IRES may function by recruitment of preassociated 80S complexes, this preassociation could hide ribosomal surfaces that serve as targets for the silencing complex on cap-dependent mRNAs that recruit the ribosomal subunits sequentially.

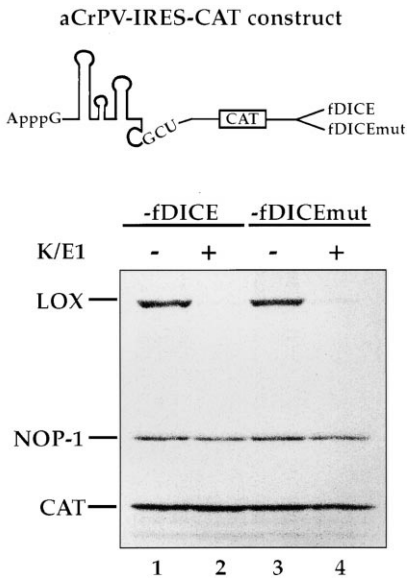

Figure 5. Translation Initiated via the CrPV-IGR-IRES Bypasses the Silencing Mechanism

(A) Schematic representation of CrPV-IGR-IRES-CAT reporter mRNAs carrying the fDICE or fDICEmut in the 3′UTR.

(B) NOP-1 and LOX fDICE mRNAs as internal negative and positive controls, respectively (lanes 1–4) were cotranslated with either CrPV-IGR-IRES-CAT-fDICE (lanes 1 and 2) or CrPV-IGR-IRES-CAT-fDICEmut mRNAs (lanes 3 and 4). Dialysis buffer (lanes 1 and 3) or hnRNPs K and E1 (lanes 2 and 4) were added to the translation reactions in rabbit reticulocyte lysate. NOP1 and LOXfDICE mRNAs were capped with a m^7GpppG cap, whereas the IGR-IRES constructs were functionally uncapped and protected at the 5′ end with an ApppG cap.

The positions of ^{35}S-Met-labeled LOX, NOP-1, and CrPV-IGR-IRES-CAT peptides are indicated on the left.

Discussion

We have investigated the question of how LOX mRNA can be translationally silenced during early erythroid differentiation. This silencing is critical, because the expression of LOX activity leads to the degradation of mitochondria, an event that characterizes late erythroid differentiation. Our results identify the step at which the LOX silencing complex disrupts the translation initiation pathway, and yield insights into the function of a 3′UTR regulatory complex.

The 3′UTR Regulatory Complex Controls 60S Ribosomal Subunit Joining

When hnRNPs K and E1 bind to the 3′UTR DICE of LOX mRNA, translation initiation is inhibited and translation-competent 80S ribosomes cannot assemble (Figures 2 and 3; Ostareck et al., 1997). By contrast, the first and often rate-limiting step in translation initiation appears to proceed with little interference, because 48S translation preinitiation complexes are formed with near normal efficiency (Figures 2C and 3D). Intuitively, one might expect that a regulatory mechanism should act on the rate-limiting step of a pathway. Indeed, the recruitment of the small ribosomal subunit has emerged as the regulated step in the vast majority of well-studied cases. However, any necessary step in a pathway, including those downstream of the normally rate-limiting one, can be targeted by inhibitory mechanisms.

The finding that 40S subunit binding is not inhibited and that a later step is affected also explains why silencing is fully operational for CSFV-IRES-mediated translation (Figure 1), which entails a different mode of 40S subunit binding to the mRNA. We have attempted to assess 40S subunit binding by the TIP assay (Muckenthaler et al., 1998), but failed to achieve sufficient signal-to-noise ratios to address the question by this technique.

In principle, the bound 40S subunit (in the form of a 43S preinitiation complex) may either be hindered to reach ("scan to") the translation initiation codon and therefore cause a failure to proceed through 60S subunit joining indirectly, or 60S subunit joining at the AUG codon may be directly inhibited. For the following reasons, we strongly favor the second possibility. First, the CSFV-IRES promotes the binding of the small ribosomal subunit at the initiation codon without prior scanning (Pestova et al., 1998). Unless one wants to postulate two different modes of action for silencing CSFV-IRES and cap-driven translation, the silencing of CSFV-IRES-mediated translation suggests that the silencing mechanism acts after the 40S ribosomal subunit is positioned at the translation initiation codon.

Second, in the presence of GMP-PNP, the hnRNP K/E1-DICE silencing complex appears to make little difference to the formation of 48S complexes that are sufficiently stable to withstand sucrose gradient sedimentation (Figures 2C and 3D). By contrast, stable 48S complexes are not recovered with the silenced mRNA when GTP hydrolysis can occur (Figures 2B and 3B). This suggests that the silencing mechanism has little effect on ribosome movement and assembly before the point of GTP hydrolysis is reached, which is thought to happen after 43S complex positioning at the AUG codon. We suggest that silencing acts coincident with or subsequent to the first GTP hydrolysis step and before a stable 80S complex is formed (Figure 6). However, it is formally possible that 48S complexes formed in the presence of GMP-PNP are intrinsically more stable even before the first GTP hydrolysis step is reached.

Third, toe printing allows direct assessment of the

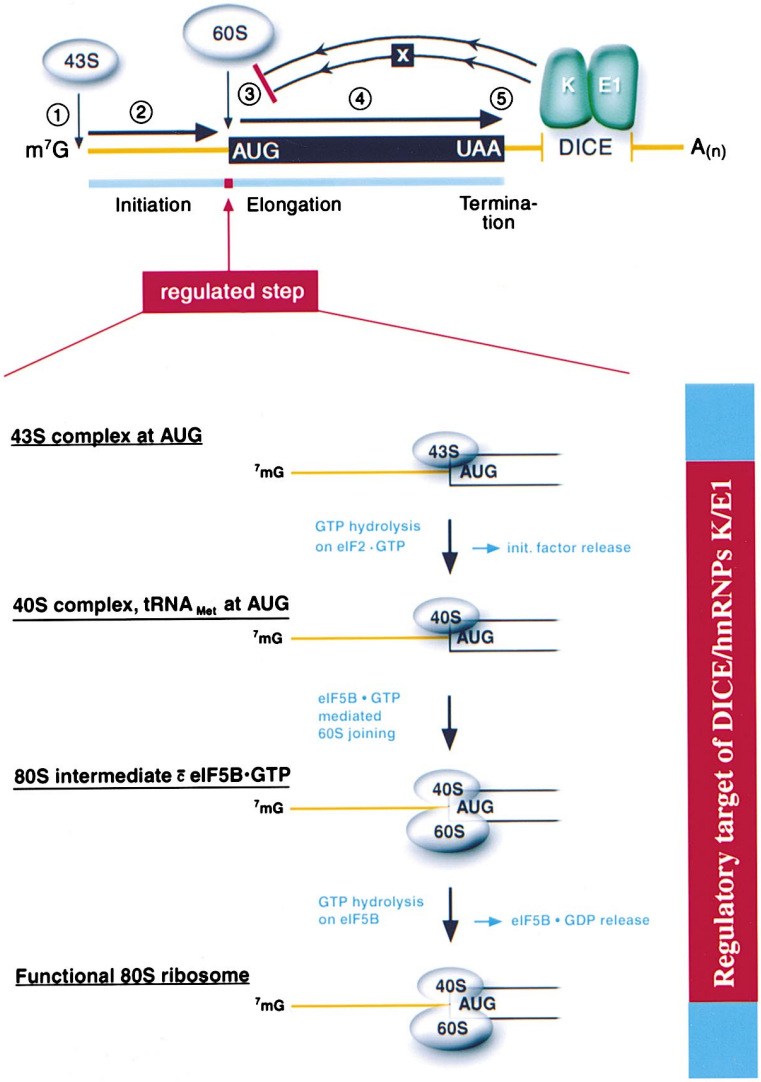

Figure 6. Mechanism of Translational Silencing by the 3′UTR hnRNP K/E1-DICE Complex

The phases of translation initiation, elongation, and termination are depicted in a stepwise fashion (blue bar), with the targeted step of 60S ribosomal subunit joining being pinpointed in red. Note that the regulatory complex may act directly or involve a silencing cofactor (white "X" in black box). The bottom half shows the two GTP hydrolysis dependent steps involved in 60S subunit joining. GTP hydrolysis stimulated by eIF5 is required for the release of eIF2 from the ternary complex (Chakrabarti and Maitra, 1991; Das and Maitra, 2000). eIF5B mediates the joining of the 60S ribosomal subunit and GTP hydrolysis is necessary for the subsequent release of eIF5B to form a stable 80S ribosome (Pestova et al., 2000).

position of ribosomal complexes at the translation initiation codon. As shown in Figure 4, ribosomal complexes reach the AUG codon of an mRNA that initiates translation by a cap-dependent "scanning" mechanism under silencing conditions, strongly supporting the conclusion that the steps prior to 60S ribosomal subunit joining can proceed with little interference from the silencing complex.

3′ to 5′ Communication and 3′UTR-Mediated Translational Control

For 3′UTR regulatory elements and binding proteins to control translation initiation, they need to exert their function, directly or indirectly, on the 5′UTR where the initiating ribosomes assemble. For such 3′ to 5′ communication, different models can be envisaged. It has been shown that the poly(A) tail can promote the recruitment of the small ribosomal subunit to the mRNA (Tarun and Sachs, 1995). This function of the poly(A) tail is mediated by interactions between the poly(A) binding protein (Pab1p/PABP) and eIF4G (Tarun and Sachs, 1996; Tarun, et al., 1997). The simultaneous binding of eIF4G to the cap binding protein eIF4E forms a loop that can physically approximate the 5′ and the 3′ end of mRNAs (Jacobson, 1996; Wells et al., 1998). A 3′UTR regulatory complex may therefore exert its function by controlling the length of the poly(A) tail, as is the case for c-mos, cyclin, and many other maternal mRNAs during early development (Sheets et al., 1994; de Moor and Richter, 1999; Barkoff et al., 2000). It may also interfere with the formation or function of the initiation-promoting loop between eIF4E, eIF4G, and PABP. The protein Maskin has been suggested to function in this way (Stebbins-Boaz et al., 1999). We think that LOX silencing works through neither of these known mechanisms, because it can operate on nonpolyadenylated and IRES-driven mRNAs. Moreover, in contrast to LOX silencing, all of these known mechanisms interfere with 40S subunit recruitment.

One can also envisage that a 3′UTR complex could control translation initiation by regulating a mechanism that "re-feeds" ribosomal subunits to the 5′ end of mRNAs after translation termination. We can exclude this model for the regulation of LOX mRNA, because

such a mechanism could by definition not operate on the first initiation cycle: for LOX mRNA, 80S ribosome formation is inhibited under conditions where elongation/termination cycles are not permitted in the presence of cycloheximide (Figures 2B and 3B). It is also possible that elements in addition to the binding site(s) for the regulatory protein(s) participate in establishing contacts between the 5′ and the 3′ end, for example by base complementarity. Such elements appear to be important for the translation of barley yellow dwarf virus RNA (Allen et al., 1999). It is unlikely that elements with base complementarity play a role in LOX mRNA regulation, because the insertion of a 38 nucleotide synthetic DICE into the 3′UTRs of different heterologous mRNAs suffices to engender translational control (Figures 1–3) (Ostareck et al., 1997).

To repress translation from the 3′ end, the silencing complex may occlude ribosomal subunit surfaces that are important for 60S joining, or inhibit the function of translation initiation factors involved in this process. Our findings with the CrPV-IGR-IRES (Figure 5) are fully consistent with the possibility that the 3′UTR LOX mRNA silencing complex (which may include a cofactor—designated "x" in Figure 6—in addition to the hnRNPs K and E1) targets one of the translation initiation factors involved in GTP hydrolysis and 60S ribosomal subunit joining (Figure 6). However, they do not exclude the former possibility (see "Results"). Future experiments will aim to directly distinguish between these two scenarios.

Experimental Procedures

Plasmids

The constructs pGEM-CAT-DICE or pGEM-CAT-NR were generated using the DICE or NR element of the LOX mRNA 3′UTR from the plasmid pBSII-SK DICE or NR (Ostareck-Lederer et al., 1994). These elements were inserted into NotI/ClaI of a synthetic oligonucleotide, which was cloned into the PstI site downstream of the CAT open reading frame. For the construction of the CSFV-IRES-CAT-DICE, CSFV-IRES-CAT-NR, CSFV-IRES-CAT-fDICE and CSFV-IRES-CAT-fDICEmut cDNAs, the CSFV-IRES including the viral translation initiation site was amplified by PCR from the plasmid pCSFV-NS′ (a kind gift from T. Pestova and C. Hellen) (Pestova et al., 1998) and inserted into the BamI/XbaI sites in the 5′UTR of the CAT open reading frame. Synthetic oligonucleotides for sORF-fDICE or sORF-fDICEmut specific sequences (5′-AGCTTGCCACCATGGACTACAA GGACGACGACGACAAGATGATGCAATGTACCTATAACCAGACCAT GTAAATCGATCCCCACCCTCTTCCCCAAGCCCCACCCTCTTCCC CAAGCCGC-3′) were cloned into the HindIII/XhoI sites of pBSII-KS, to create an open reading frame of 19 amino acids, before the fDICE or fDICEmut 3′UTR elements. The plasmid short CAT (sCAT) represents the CAT sequence, lacking the BsmI/ScaI fragment (428 nt). The element fDICE was cloned into PstI/SphI sites 3′ of the sCAT open reading frame. For the plasmid CrPV-IGR-IRES CAT, the IGR-IRES of the cricket paralysis virus (CrPV) (a kind gift of J. E. Wilson and P. Sarnow) (Wilson et al., 2000b) was cloned between BamHI/XbaI sites 5′ to the CAT open reading frame. The elements fDICE or fDICEmut, respectively, were cloned as for the CSFV-IRES CAT constructs (see above). The cloning of the LOX fDICE (Ostareck et al., 1997) and pGEM CAT as well pBSII-SK NOP-1 cDNAs have been described (Stripecke and Hentze, 1992). All plasmid constructs were verified by DNA sequencing.

Expression of Recombinant Proteins

The expression and purification of recombinant proteins hnRNP E1 and hnRNP K has been described (Ostareck et al., 1997).

In Vitro Transcription and Cell-Free Translation

Transcripts were generated (capping efficiency of >95%) as described (Ostareck et al., 1997). mRNA concentrations and integrity were assessed by trace labeling and agarose gel electrophoresis. For translation initiation reactions, capped ^{32}P-labeled mRNAs were transcribed and purified as above (specific activity 1.2×10^7 cpm/μg). For cell free translations (12 μl reaction), rabbit reticulocyte lysate (RRL) was prepared as described by Jackson and Hunt (1983) with modifications (Ostareck et al., 1997). In Figure 1B, the reactions contained 1μl of m^{7}GpppG capped mRNAs (3.8 ng NOP-1, 1 ng of CAT-DICE and CAT-NR, and 15 ng CSFV-IRES-CAT-DICE, -NR, -fDICE, and -fDICEmut), as indicated. In Figure 5B, m^{7}GpppG capped mRNAs (20 ng LOX fDICE and 5 ng NOP1) and 100 ng ApppG-capped CrPV-IGR-IRES-CAT-fDICE or -fDICEmut mRNAs were translated in the presence of 154 mM potassium acetate. Where indicated, 30 ng (Figure 1B) or 100 ng (Figure 5B) of hnRNPs K and E1 were added to the mRNA as a mixture (3:1 molar ratio) and incubated on ice prior to the translation reaction.

Translation Initiation Assays and Sucrose Gradient Analysis

RRL was preincubated with 0.5 mM cycloheximide for 3 min at 30°C (Ostareck et al., 1997). Where indicated, 2 mM GMP-PNP was incubated alongside with cycloheximide. In Figures 2 and 3, 8 ng of ^{32}P-labeled sORF-fDICE or -fDICEmut mRNA were incubated for 10 min at 4°C with 75 ng of recombinant hnRNPs K and E1 (3:1) or with dialysis buffer. The initiation reaction (48 μl) at 30°C was stopped by addition of ice-cold dilution buffer after 5 min (Figure 2) or 30 s, 2, 5, or 10 min, respectively (Figure 3). Initiation complexes were resolved on linear 5%–25% sucrose gradients (Ostareck et al., 1997). Sucrose fractions (250 μl each) were collected from the bottom of the gradient and were analyzed by scintillation counting.

Toe Printing

For toe printing in RRL, 200 ng of m^{7}GpppG capped sCAT-fDICE mRNA were used, preincubated for 10 min on ice with dialysis buffer or an 8-fold molar excess of hnRNPs K and E1 (3:1). This mRNA was added to RRL preincubated at 30°C for 3 min with 0.5 mM cycloheximide and, where indicated, 1 mM m^{7}GpppG, or 1.5 mM GMP-PNP. Initiation complexes were assembled for 5 min at 30°C. 5 pmol of ^{32}P-labeled RT-2 primer were hybridized to the sCAT-fDICE mRNA in the 12 μl reaction on ice for 3 min. For primer extension, the reaction was diluted 20-fold with buffer containing 0.5 mM cycloheximide, 7 mM magnesium acetate, 100 mM potassium acetate, 0.5 mM dATP, dCTP, dGTP, and dTTP, 20 mM Tris (pH 7.4), 2 mM DTT, and 0.25 U/μl AMV-RT and incubated at 30°C for 10 min. After proteinase K treatment, the reaction was phenol/chloroform extracted and cDNA products were precipitated and analyzed on an 8% polyacrylamide sequencing gel. cDNA products were compared with a dideoxynucleotide sequencing ladder obtained using the same primer and sCAT-fDICE plasmid DNA.

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