Temporal Translational Control by a Metastable RNA Structure*

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Programmed cell death by the hok/sok locus of plasmid R1 relies on a complex translational control mechanism. The highly stable hok mRNA is activated by 3′-end exonucleotical processing. Removal of the mRNA 3′ end releases a 5′-end sequence that triggers refolding of the mRNA. The refolded hok mRNA is translatable but can also bind the inhibitory Sok antisense RNA. Binding of Sok RNA leads to irreversible mRNA inactivation by an RNase III-dependent mechanism. A coherent model predicts that during transcription hok mRNA must be refractory to translation and antisense RNA binding. Here we provide genetic evidence for the existence of a 5′ metastable structure in hok mRNA that locks the nascent transcript in an inactive configuration in vivo. Consistently, the metastable structure reduces the rate of Sok RNA binding and completely blocks hok translation in vitro. Structural analyses of native RNAs strongly support that the 5′ metastable structure exists in the nascent transcript. Further structural analyses reveal that the mRNA 3′ end triggers refolding of the mRNA 5′ end into the more stable tac-stem conformation. These results provide a profound understanding of an unusual and intricate post-transcriptional control mechanism.

RNA molecules fold into highly ordered structures essential to their diverse biological functions. Accordingly, the question of how the linear sequence of ribonucleotides dictates the overall folding of an RNA has received much attention (1–6). Folding of RNA, whether occurring from a denatured state or sequentially in concomitance with its synthesis, involves the formation of a number of hierarchically ordered intramolecular interactions leading to increasing levels of structural organization. In both cases, structural rearrangements of kinetically favored folding intermediates may occur before the thermodynamically most stable conformation is reached (7, 8). In the course of folding, RNA molecules face the risk of being trapped in nonequilibrium conformations. Because of the high thermodynamic stability of RNA secondary structures, rearrangement of such nonequilibrium conformations can constitute a substantial energy barrier, thus leading to kinetic trapping of the RNA in thermodynamically suboptimal conformations termed metastable structures (9–11). Metastable folding intermediates have proved important to a number of biological processes including plasmid replication (12), replication of RNA by QF replicase (13, 14), human immunodeficiency virus-1 RNA export to the cytoplasm (15), ribozyme activity (16–20), and viroid replication (21–23).

The hok/sok locus of plasmid R1 mediates plasmid maintenance by the killing of plasmid-free cells, also termed post-segregational killing (PSK)† (24). The PSK mechanism, which restricts synthesis of Hok toxin to newborn plasmid-free cells, is controlled entirely at the post-transcriptional level. The hok/sok locus, presented schematically in Fig. 1, specifies two transcripts: the toxin-encoding hok (host killing) mRNA and the labile antisense inhibitor Sok RNA (suppression of killing). mok (mediator of killing) is a reading frame, the translation of which is required for the translation of Sok (because of translational coupling of hok to mok). Sok RNA is complementary to the hok mRNA 5′ end and inhibits hok translation indirectly by occluding the mok ribosome-binding site (25).

Full-length versions of hok mRNA (1 and 2) are unusually stable because of extensive secondary structure formation and specific base pairings between their 5′ and 3′ ends (26). The structure of hok-2 mRNA is shown in Fig. 2B. The 5′-to-3′ base pairing in the full-length molecules yields compact structures, and hok mRNA-1 and -2 are inactive with respect to translation and antisense RNA binding (25–28). Translation of hok mRNA-2 is activated by slow 3′-exonucleotical removal of the fbi (fold-back inhibition) element (Fig. 2B). The 3′ processing releases the 5′ tac (translational activator) element and thereby triggers a series of structural rearrangements prerequisite for translation and rapid Sok RNA binding (25–29). Thus, the activated refolded hok mRNA contains the energy-rich tac stem and the antisense RNA target hairpin (Fig. 2C), the latter of which is required both for translation and rapid antisense RNA binding (25, 26, 29, 30).

The presence of tac in the mRNA 5′ end suggested the existence of a regulatory element that would prevent initiation of translation during transcription. Nucleotide covariations in the aligned family of hok mRNAs indicated that the mRNAs could form small metastable hairpins at their 5′ ends (30). However, the function of these hairpins is not known. Here we present evidence that hok mRNA specifies a structure that simultaneously prevents antisense RNA binding and synthesis of Hok toxin during transcription. This molecular safeguard consists of two small metastable hairpins at the mRNA 5′ end (Fig. 2A). During transcription, the metastable hairpins prevent formation of the tac stem and ultimately inhibit formation of the target hairpin. Our data indicate that the metastable hairpins exist until completion of transcription and that their disruption is triggered by the fbi sequence at the mRNA 3′ end. Thus, we reveal here that hok mRNA follows a specific folding pathway that seems to have evolved to allow translation of hok in plasmid-free cells only.

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‡ The abbreviations used are: PSK, post-segregational killing; fbi, fold-back inhibition; tac, translational activator; PCR, polymerase chain reaction; DTT, dithiothreitol; SD, Shine-Dalgarno sequence.
Programmed Cell Death by hok/sok of Plasmid R1

**A:** nascent hok transcript

**B:** full-length hok mRNA

**C:** truncated, refolded hok mRNA

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**EXPERIMENTAL PROCEDURES**

**Enzymes and Chemicals—**Ampicillin was added to a concentration of 30 μg/ml for mini-R1 plasmids and 100 μg/ml otherwise. Rifampicin (Ciba-Geigy) was added to cells prior to RNA extraction at a concentration of 250 μg/ml. Restriction endonucleases were purchased from Roche. All other enzymes were purchased from Promega.

**Bacterial Strains—**The partially Hok-resistant Escherichia coli mutant strain NWL37 (HokR, CmlR, KanR) (31) was used for the establishment of plasmids carrying mutated hok/sok loci. The E. coli K-12 strain CSH50 (Δlac pro) was used for the establishment of plasmid stability tests (33) and rifampicin induction experiments. Both strains were cultured in Luria-Bertani broth (34) at 37°C.

**Plasmids—**Plasmid pBR322 carries the 580-base pair wild-type hok/sok locus from plasmid R1 cloned between the EcoRI and BamHI sites of pBR322. Plasmids pJM210 (U21A), pJM216 (A9U), and pJM218 (U21A/A9U) are mutant derivatives of pBR322. The probe plasmid, pGEM342, carries the hok/sok 240 base pairs downstream Sau3A-EcoRI fragment cloned under the T7 promoter of pGEMblue.

**Site-directed Mutagenesis—**The U21A, A9U, and U21A/A9U mutations were introduced using double PCR as described in Ref. 35 using T7-pPerfect, 5'-CACGT-GGCAGAAAGCC; and A9U, 5'-CATG-GGCAGAAAGCC. PCR templates for Sok RNA synthesis were T7-sok wild type, 5'-GTAAAACGACGGCCAGT; EcoRI clockwise, 5'-GTATCAGAGGCCCCTTTCG; U21A, 5'-CACTGCATGGCCAGAAGCC; and A9U, 5'-GGCAGAAGCCACAGGCGCGGCAG (mutant nucleotides are printed in bold face).

**Total RNA Preparation and Northern Transfer Analysis—**The preparation of total RNA from E. coli and Northern transfer analysis were performed as described previously (27).

**Synthesis of hok mRNA and Sok RNA in Vitro—**The preparation and purification of uniformly 32P- or 3H-labeled RNA molecules were performed as described by Franch and Gress (26). The RNA species were synthesized using T7 RNA polymerase and templates generated by PCR. In all PCRs, pBR322 was used as the template. The T7 promoter region was 20 primer, 5'-GTTAAAACGACGGCCAGT; EcoRI clockwise, 5'-GTATCAGAGGCCCTTTCG; U21A, 5'-CACTGCATGGCCAGAAGCC; and A9U, 5'-GGCAGAAGCCACAGGCGCGGCAG (mutant nucleotides are printed in bold face).

**Fig. 1.** Genetic organization and regulatory elements of the hok/sok locus from plasmid R1. The arrows pointing right indicate the mRNAs encoded by hok/sok: the two full-length molecules, hok mRNA-1 and hok mRNA-2, and truncated (Tr) hok mRNA. An arrow pointing left indicates Sok antisense RNA. The positions of the 5’ tac and the 3’ fbi elements are indicated. Open boxes in the mRNAs indicate the antisense RNA target. Larger boxes represent the overlapping mok and hok reading frames.

**Fig. 2.** Folding pathway of hok mRNA. A, structure of the nascent hok transcript as predicted by the genetic algorithm (43, 44). Experimental evidence for the metastable hairpins in native hok mRNA was obtained in this work (Fig. 6A) and in shorter hok mRNA fragments as described in Ref. 40. The U21A and A9U substitutions are indicated by opposing arrows. An arrow pointing down indicates the nucleotide that is complementary to the 5’ nucleotide of Sok RNA. Translational start signals of hok and mok are boxed. A solid line indicates the DNA oligonucleotide complementary to the mok ribosome-binding site that was used for structure probing. B, structure of the full-length hok mRNA that contains the ibi-tac interaction and the top of the tac stem. The U21A and A9U substitutions are indicated by opposing arrows. An arrow pointing down indicates the nucleotide that is complementary to the 5’ nucleotide of Sok RNA. Translational start signals of hok and mok are boxed. A solid line indicates the DNA oligonucleotide complementary to the mok ribosome-binding site that was used for structure probing. The structures shown in B and C were confirmed by phycogenetic comparisons and structural and genetic analyses (25, 26, 30).
sequen SDS-polyacrylamide gel electrophoresis was carried out as described by Franch and Gerdes (26).

In Vitro Binding Reactions between hok mRNA and Sok RNA—Binding experiments were performed as described previously (25).

Coupled In Vitro Transcription/RNase H Cleavage Assay—DNA templates for in vitro transcription were prepared by PCR. The PCR templates were identical to those used for the purification of labeled in vitro transcripts (as described above). PCR products were purified by a PCR purification kit (Qiagen), extracted with phenol/CHCl₃, and precipitated with NH₄OAc and ethanol. Transcription assays were carried out by incubation of 5 pmol of DNA template with 10 mCi of [35S]-CTP, 10 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 16 μM of CTP, 10 μCi of [α-32P]-CTP, 125 mM GTP and UTP, 18 units of RNA-guard (Amersham Pharmacia Biotechn), and 20 units of T7 RNA polymerase for 8 min at room temperature. After incubation, 125 mM ATP was added to the preincubation mixture for initiation of transcription. Four-microliter aliquots were removed for RNase H probing at various time points after the initiation of transcription. Probing mixtures contained 4 μl of transcription mix, TMK-glutamate (see above) supplemented with 5 mM DTT, 10 pmol of DNA oligo, and 1 unit of RNase H. After 30 s of probing, samples were frozen in dry ice/ethanol, extracted with phenol/CHCl₃, and precipitated. The transcripts were redissolved in formamide dye and analyzed by autoradiography on a 6% denaturing acrylamide gel. The oligonucleotide sequence used was 5'-CTCTGTTGGTG.

Results

The U21A Mutation in hok mRNA Increases Sok Antisense RNA Binding in Vivo—Based on folding simulations and phylogenetic analyses of the hok family of mRNAs, two hairpin structures were proposed to form at the hok mRNA 5' end (30). The secondary structure of the nascent hok transcript is shown in Fig. 2A. By completion of transcription the hairpins must give way for the thermodynamically more stable structures, characteristic of the full-length molecule shown in Fig. 2B. Notably, the full-length molecule contains the 5'-3' interaction and the top of the long tac stem. The U21A mutation that destabilizes the proposed metastable structure (Fig. 2A) while increasing the energy of the alternative tac stem (Fig. 2C) is expected to increase the rate of the structural transition from the metastable structure to the tac stem (see Fig. 2, A and C). Along with the U21A mutation, we introduced the second-site A9U mutation, which restores the possibility of forming the metastable hairpin. The effects of U21A and A9U mutations on the stability and processing pattern of hok mRNA in vivo was assayed by Northern analysis. In the wild-type case (Fig. 3A, first panel), the processing pattern is explained as follows (24): truncated hok mRNA is slowly and constitutively generated by exonucleolytic processing of hok mRNA-2. In the presence of Sok RNA, truncated hok mRNA is removed rapidly by RNase III cleavage of the hok/sok duplex RNA. Therefore, truncated hok mRNA is not observed in cells growing in steady state (i.e. before the addition of rifampicin in Fig. 3A). However, after rifampicin, Sok RNA decays rapidly. This in turn allows for accumulation of the stable truncated hok mRNA.

The U21A mutation dramatically reduced the levels of both full-length molecules (Fig. 3A, second panel) and inactivated the ability of the system to mediate plasmid stabilization by the PSK mechanism (data not shown). During steady-state cell growth, a band corresponding to the major product of Sok-mediated RNase III cleavage of hok mRNA is visible, indicating that the predominant fraction of the molecules is degraded by RNase III (-2 min sample in second panel). This in turn indicates that mutated hok mRNA binds Sok RNA prematurely or, less likely, that Sok RNA exhibits enhanced binding to the full-length RNAs.

We then investigated whether disruption of the metastable structure by the U21A mutation could be back-complemented by the U9A mutation (Fig. 2C). As seen from Fig. 3A (fourth panel), the U21A/A9U double mutation restored the processing pattern to that of the wild-type mRNA. This provides genetic evidence for the existence of the 5' metastable hairpin. The A9U single mutation had no effect on the mRNA processing pattern, probably because it simultaneously reduces the energy of the metastable and tac stems (see "Discussion").

To further investigate the function of the metastable structure in vivo, we analyzed hok mRNA band patterns in the absence of Sok RNA (Fig. 3B). In the wild-type case, truncated hok mRNA is now present in steady state, because it cannot be removed by antisense RNA-mediated RNase III cleavage of hok mRNA, indicating that the predominant fraction of the molecules is degraded by RNase III (-2 min sample in second panel). This in turn indicates that mutated hok mRNA binds Sok RNA prematurely or, less likely, that Sok RNA exhibits enhanced binding to the full-length RNAs.

Discussion

The Metastable Structure Reduces the Rate of Antisense RNA Binding in Vitro—Two mutant mRNAs were constructed (Fig. 4). In one RNA termed "hok super-metastable" mRNA, the metastable hairpins were forced by the introduction of five base changes in the 5' stem. In the second RNA termed "hok super-tac," the tac stem was forced by five different base changes (Fig. 4). The secondary structures of the forced mutant RNAs were confirmed experimentally (data not shown). Sok RNA and variants of truncated hok mRNA were synthesized in vitro, gel-purified, and renatured.

Fig. 3. In vivo processing patterns of wild-type and mutated hok mRNAs shown by Northern analysis. A. sok+ plasmids; B. sok− plasmids. Total RNA was prepared from cells (C5H50) grown in LB medium. The different strains contained pBR322 derivatives with wild-type or mutated hok/sok loci as indicated above each panel. For plasmid designations, see "Experimental Procedures." The positions of full-length hok mRNAs-1 and -2 and the truncated (Tr) mRNA as well as the major RNase III cleavage (Cl) product are indicated. Time points of sampling relative to the addition of rifampicin are indicated above each lane. wt, wild type.

Experimental Procedures.
super-metastable

U   U   C
U   C   G
G   C   A
A   G   T
G   G   A
A   C   G
G   C   G
G   C   C
U   U   G
5'   C   G   G   G   G   G   G   G   G   G   G   G   A   A   A   A   A

super-tac

G   U   G   C   C
U   G   G   U   U   G
G   G   A   A   A   A   A
A   G   U   C   C
C   G   A   A   A   A
G   U   G   G   U
5'

Fig. 4. Secondary structures of forced mutant hok mRNAs. Shown are the secondary structures of hok mRNA 5' ends containing the super-metastable and super-tac mutations that were used for in vitro analyses. Changed nucleotide residues are underlined.

Using an in vitro binding assay (38), the apparent second-order binding-rate constants ($K_{app}$) of Sok RNA association with truncated wild-type and mutant hok mRNAs were determined (Table I). Sok RNA bound with similar high rates to truncated wild-type and super-tac mRNAs. However, the binding rate was reduced ~10-fold in the case of the super-metastable hok mRNA. Thus in this assay the forced metastable structure reduced antisense RNA binding significantly. This is consistent with the notion that the super-metastable structure prevents the formation of the antisense RNA target hairpin (Fig. 2C). In contrast, Sok RNA bound rapidly to both purified wild-type and super-tac hok mRNAs, indicating that these molecules contain the antisense RNA target hairpin that mediates that highest rate of antisense binding (25, 39). Note these molecules contain the antisense RNA target hairpin that probably because the mutation destabilizes the tac stem (26) (Fig. 3C).

The Metastable Structure Inhibits Translation—To test the effect of the metastable structure on hok translation, wild-type and mutant hok mRNAs were translated in a cell-free S30 extract (37). When gel-purified and renatured, the wild-type truncated hok mRNA was translated efficiently (Fig. 5, +, upper panel). This result is consistent with the finding that renatured truncated hok mRNA folds into the translatable configuration that contains the energy-rich tac stem (26, 39). Note that the molecules used above were denatured and renatured during their purification.

To examine if this was also the case for the native molecule, transcripts were synthesized in vitro and added directly to the S30 extract without denaturation and renaturation. Strikingly, truncated wild-type hok mRNA in its native form was not translated at all (Fig. 5). Native truncated hok mRNA carrying the forced metastable structure was also translated very inefficiently. In contrast, the native forms of hok mRNAs carrying the super-tac and U21A mutations were translated efficiently. Truncated hok mRNA carrying the ASU was not translated, probably because the mutation destabilizes the tac stem required for translation. As expected the two full-length hok mRNAs also were not translated (Fig. 5). These results indicate that mutations or conditions that favor the metastable structure prevent translation, whereas the presence of the tac stem allows translation.

Native hok mRNA Contains the Metastable Hairpins—An important inference from the above-described results was that gel-purified truncated hok mRNA should contain the tac stem, whereas the native form of the molecule should contain the metastable structure. To investigate this directly, we performed a structural analysis. As seen in Fig. 6A, the RNase T2 cleavage patterns (T2 cleaves 3' of unpaired nucleotides) of the two forms of the RNA are strikingly different. The purified form has prominent bands corresponding to the tac-stem loop (tac in Fig. 6A), whereas these bands are reduced in the native isoform of the mRNA. In contrast, the native isoform exhibits enhanced cleavage at bases corresponding to the loops of the metastable hairpins (m1 and m2 in Fig. 6A). The clear differences between the RNAs indicate that the metastable hairpins exist in truncated hok mRNA. Leaving the native truncated hok mRNA at room temperature for 30 min before probing does not change the probing pattern (data not shown), indicating that this configuration is stable throughout the probing experiment.

The 5' Metastable Structure in Native hok mRNA Is Thermodynamically Stable—To study the refolding kinetics of native hok mRNA, a coupled transcription/RNase H-probing time-course assay was conducted (Fig. 7; see “Experimental Procedures”). Native wild-type and mutated truncated hok mRNAs were generated with T7 RNA polymerase and structure-probed with RNase H using an oligonucleotide complementary to the

### Table I

<table>
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<td>super-tac</td>
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</tr>
<tr>
<td>super-metastable</td>
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### Figures

**Fig. 4.** Secondary structures of forced mutant hok mRNAs. Shown are the secondary structures of hok mRNA 5' ends containing the super-metastable and super-tac mutations that were used for in vitro analyses. Changed nucleotide residues are underlined.

**Fig. 5.** In vitro translation of wild-type (wt) and mutated native hok mRNA molecules. RNAs were synthesized in vitro using T7 RNA polymerase and subjected to in vitro translation in an E. coli S30 extract. Proteins were labeled by incorporation of $^{35}$Smethionine and separated for autoradiography by Tricine-SDS-polyacrylamide gel electrophoresis (upper panel). The type of RNA molecule added to each reaction is indicated above each lane. +, indicates translation of purified and renatured truncated hok mRNA. The relative amount of RNA added to each translation reaction was visualized by Northern blotting (lower panel, see “Experimental procedures”). Protein samples were normalized such that the protein levels in the upper panel reflect the specific translational activity of each mRNA.
region of the mok ribosome-binding site (see Fig. 2, A and C). Samples were withdrawn from the transcription mix at various time points after initiation to monitor structural rearrangements. Native hok mRNA containing the forced tac stem exhibited efficient cleavage (Fig. 7, third panel), indicating that in this RNA the mok ribosome-binding site exists in an open configuration. This is consistent with the translatability of the RNA (Fig. 5). In contrast, hok mRNA containing the forced metastable structure was not cleaved at all (Fig. 7, second panel), indicating that the mok ribosome-binding site is in a closed configuration. Again, the closed structure is consistent with the observed lack of translation (Fig. 5). Note that the mok SD region is located 70 nucleotides downstream of the mutational changes in the RNAs, showing that the mutations impose long-range structural changes in the RNAs.

Most importantly, wild-type native hok mRNA exhibited the same RNase H cleavage pattern as the RNA containing the forced metastable structure, and the pattern did not change in time (Fig. 7, first panel). These results support that the metastable structure exists stably in native truncated hok mRNA and that it keeps the mok SD region in a closed configuration.

The 3' End of hok mRNA Triggers Refolding of the mRNA 5' End—Fig. 6B shows a structural analysis of purified versus native full-length hok mRNA-1. Both the purified and native isoforms were cleaved at positions corresponding to the loop of the tac stem, indicating that the native molecule contains this structure. This stands in contrast to truncated wild-type hok mRNA, in which case the native form exhibited a cleavage pattern compatible with the metastable structure (Fig. 6A). Thus, the presence of the 3' hok target loop eliminates the existence of the metastable hairpins. By inference, this suggests that the 3' hok target loop triggers the disruption of the metastable hairpins in the native full-length mRNA.

**FIG. 6.** Secondary structure probing of native and purified hok mRNAs. Native and purified truncated (A) and full-length (B) hok mRNAs were treated with RNase T2. After treatment with RNase T2, the cleavage sites were visualized by primer extension using a 5'-labeled DNA primer (denoted hokA) complementary to a region in the hok reading frame. The RNA sequence reaction was accomplished with the same DNA primer as described under “Experimental Procedures.” Secondary structure elements mentioned in the text are indicated. m1 and m2 indicate cleavages in the loops of the metastable hairpins; tac denotes cleavages in the loop of the tac hairpin; target denotes cleavages in the antisense RNA target loop. Strong cleavages, consistent with the presence of the target loop (with the U-turn), are seen in the purified truncated RNA. Native truncated RNA and both full-length isoforms are devoid of such cleavages.

**FIG. 7.** Coupled in vitro transcription/RNase H cleavage assay. RNAs were synthesized in vitro with T7 RNA polymerase. The transcription reaction was initiated at time 0 by the addition of ATP. Samples of uniformly labeled hok mRNA were withdrawn from the transcription mix at the indicated time points and subsequently probed using SD-end oligo and RNase H. C, control sample removed after 20 min of transcription and probed in the absence of oligonucleotide. M, control reaction purified, uniformly labeled truncated wild-type hok mRNA was cleaved using the SD-end oligo and RNase H. Intact transcripts and cleavage products are indicated by arrowheads. wt, wild type.
The in vivo results presented in Fig. 3 corroborate the proposed function of the metastable structure. To obtain more direct evidence, we employed in vitro techniques. Enforcement of the metastable structure of hok mRNA led to a 10-fold decrease in the in vitro Sok RNA binding rate (Table I). The enforced metastable structure precludes the formation of the tac and target stem loops (Figs. 2A and 6A). The target hairpin contains a specialized structure (the U-turn) that is required for rapid antisense binding (39). As in tRNA, the U-turn exposes three bases in the target loop, thereby enhancing the Sok RNA binding rate ~10-fold. Thus, if the target hairpin cannot form, the U-turn structure cannot form either, and rapid antisense binding is prevented. This explains the slower binding rate for a target RNA that contains the enforced metastable structure. On the other hand, purified wild-type and super-metastable hok RNAs bound Sok RNA rapidly, indicating that both of these RNAs are in the tac-stem form (Table I).

Previously, we found that gel-purified truncated hok mRNA was translated efficiently, whereas full-length hok mRNA-1 and -2 were translated poorly (25, 26, 28). Here we expand our analyses using native RNAs that were not denatured and re-natured before being subjected to translation (Fig. 5). Native full-length mRNA-1 and -2 were translated poorly, consistent with the finding that native full-length mRNA-1 did not contain the target stem-loop known to be present in the active metastable structure. This is consistent with the native full-length RNAs being in the closed configuration containing the fbi-tac interaction but lacking the target stem-loop (Figs. 2B and 6B).

Gel-purified truncated hok mRNA was translated efficiently. In contrast, the native isoform of the RNA was not translated at all (Fig. 5). Structure mappings of the two isoforms showed patterns consistent with the metastable structure being present in the native RNA and the tac/target-stem-loops in the purified species (Fig. 6A). Thus, we conclude that the metastable structure prevents translation in native truncated hok mRNA. This conclusion is corroborated by translational analysis of the series of mutant mRNAs (Fig. 5). Mutations favoring the metastable structure (super-metastable and U21A/A9U) reduce translation, whereas mutations favoring the tac/target configuration (super-tac and U21A) favor translation. The native mRNA carrying the A9U mutation was translated at a lower rate than the one carrying U21A. Both mutations disrupt the metastable structure, but only U21A increases the stability of the tac stem. The low translation rate conferred by A9U may be caused by secondary effects on tac-stem stability (e.g. disruption of a possible noncanonical GA/AG base pairing at the bottom of the tac stem, see Fig. 2C).

Native and purified full-length hok mRNA-1 was also structure-probed (Fig. 6B). In this case, the cleavage patterns were indistinguishable. The cleavage pattern is consistent with previous structure-probing of purified RNAs (25) and indicates that the native full-length molecules do not contain the metastable structure but rather the top of the tac stem (Fig. 2B). Thus, in contrast to native truncated hok mRNA, native full-length molecules do not exhibit a cleavage pattern compatible with the metastable configuration (Fig. 4, A and B). However, both molecules are in closed configurations lacking the anti-sense target hairpin. A reasonable inference from these results is that the 3’-end fbi element triggers disruption of the metastable structure by forming the fbi-tac interaction (the transition from A to B in Fig. 2). In turn, this triggers formation of the top of the tac stem in the full-length molecule. Hence, in the course of transcription, one inactive form of the mRNA (containing the metastable structure) is replaced actively by the formation of a second inactive form (the full-length transcript). In other words, co-transcriptional repression of hok translation by the metastable structure in vivo does not rely on refolding kinetics but rather assumes a “lock and key” mechanism, in which the fbi element constitutes the key for unlocking the metastable structure. This is in keeping with hok/sok biology, because spontaneous refolding of the 5’ end into a translatable conformation could be lethal to the cell.

Because of the considerable thermodynamic stability of the two metastable hairpins, refolding is unlikely to involve complete unwinding of the two hairpins prior to the formation of thermodynamically more stable structures. Such a transition would require a large activation energy and thus becomes extremely slow at physiological temperatures. Rather the refolding occurs through a number of folding intermediates by an RNA strand exchange mechanism in which one secondary structure is formed at the expense of another. Such mechanisms have been proposed to account for the rapid interchange of RNA secondary structures in Leptomonas collosoma spliced leader RNA (41) and the PI–1/F1 region of Tetrahymena group I intron (17). One possibility is that the extreme 5’-end nucleotides serve as a toehold for nucleation of fbi-tac formation and concomitant unwinding of the metastable hairpin I. Analysis of a hok G30C mutant, however, suggests that this is not the case. The G30C mutation, which creates a base pair with the extreme 5’-end guanosine, has no effect on PSK, indicating that refolding is unaffected by this substitution (data not shown). Alternatively, refolding can be nucleated by the formation of a pseudoknot intermediate by long range interaction between loop nucleotides (U13-C16) of metastable hairpin I and their downstream complementary subset (G60-A63). Such an intermediate structure has been proposed for the spliced leader RNA (42).

The present identification of a metastable structure formed during transcription extends the concept of hok mRNA folding dynamics to involve two refolding events that are separated in time. Initially, during early stages of transcription two metastable hairpins are formed in the very 5’ end of the mRNA. As the rate of RNA secondary structure formation exceeds that of RNA synthesis by orders of magnitude, their formation is favored kinetically. By sequestering the 5’-end tac element, the metastable hairpins facilitate subsequent formation of downstream inhibitory structures, protecting the RNA against premature degradation and the cell against Hok-toxin synthesis. Upon completion of transcription, the long range fbi-tac interaction together with the partial tac stem replace the metastable structure. Eventually, slow constitutive processing of the full-length mRNA leads to the second refolding event, which produces the active version of the hok mRNA (Fig. 2C). Finally, depending on the presence of Sok RNA, the mRNA is either translated or inactivated.

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