Programmed Cell Death by hok/sok of Plasmid R1: Processing at the hok mRNA 3'-end Triggers Structural Rearrangements that Allow Translation and Antisense RNA Binding

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The hok/sok locus of plasmid R1 mediates plasmid stabilization by killing of plasmid-free cells. The locus specifies two RNAs, hok mRNA and Sok antisense RNA. The post-segregational killing mediated by hok/sok is governed by a complicated control mechanism that involves both post-transcriptional inhibition of translation by Sok-RNA and activation of hok translation by mRNA 3' processing. Sok-RNA inhibits translation of a reading frame (mok) that overlaps with hok, and translation of hok is coupled to translation of mok. In the inactive full-length hok mRNA, the translational activator element at the mRNA 5'-end (tac) is sequestered by the fold-back-inhibitory element located at the mRNA 3'-end (fbi). The 5' to 3' pairing locks the RNA in an inert configuration in which the SDmok and Sok-RNA target regions are sequestered. Here we show that the 3' processing leads to major structural rearrangements in the mRNA 5'-end. The structure of the refolded RNA explains activation of translation and antisense RNA binding. The refolded RNA contains an antisense RNA target stem-loop that presents the target nucleotides in a single-stranded conformation. The stem of the target hairpin contains SDmok and AUGmok in a paired configuration. Using toeprinting analysis, we show that this pairing keeps SDmok in an accessible configuration. Furthermore, a mutational analysis shows that an internal loop in the target stem is prerequisite for efficient translation and antisense RNA binding.

Keywords: Sok; hok; antisense RNA; translational initiation; RNA rearrangements

Introduction

The hok/sok system of plasmid R1 codes for two RNAs, hok mRNA and Sok antisense RNA (Gerdes et al., 1988, 1990). The mRNA encodes two reading frames, denoted hok (host killing) and mok (modulation of killing), that overlap extensively (Figure 1). Translation of hok is coupled to translation of mok (Thisted & Gerdes, 1992). Sok-RNA represses translation of mok via binding to the translational initiation region (TIR) of mok (Thisted et al., 1994a). Therefore, Sok-RNA regulates hok translation indirectly through mok. The 64 nt Sok-RNA consists of a stem-loop with an 11 nucleotide 5' single-stranded extension (Figure 2A). The 5' extension is complementary to the part of the mok TIR that we denote sokT (Figure 2) and is responsible for the initial recognition reaction between the RNAs (Thisted et al., 1994a). After initial recognition, more extensive duplex formation progresses from the Sok-RNA 5'-end by a zipperping-like mechanism. Subsequently, such RNA duplexes are cleaved by RNase III (Gerdes et al., 1992). Thus, binding of Sok-RNA to hok mRNA occurs by a one-step binding mechanism similar to the RNA-IN/RNA-OUT pairing in Tn10 (Kittle et al., 1989; Case et al., 1989).

The complex RNA metabolism prerequisite for activation of hok translation in plasmid-free cells is described in detail in the accompanying paper (Gultyaev et al., 1997) and is briefly outlined...
below. We showed previously that hok mRNA exists in two variants in vivo (Gerdes et al., 1990; Thisted et al., 1994a). A truncated, translationally active mRNA is generated by slow exonucleolytic removal of 40 nt from the 5' end of the full-length mRNA. Thus, the full-length mRNA acts as a reservoir from which the active mRNA is continuously generated. In plasmid-carrying cells, the presence of Sok-RNA prevents translation of the truncated mRNA. However, in plasmid-free cells, in which Sok-RNA has decayed, the continuous slow processing of the full-length mRNA leads to accumulation of the translatable truncated mRNA. Eventually, this leads to synthesis of the toxic Hok protein, and killing of the plasmid-free cells ensues.

Recently, we showed that the stable, inactive hok mRNA is compactly folded with only a few single-stranded regions, apart from loops (Franch & Gerdes, 1996). A genetic analysis established that the full-length mRNA contains a peculiar 5' to 3' long-range pairing in which the very first nucleotides pair with the very last ones (Figure 2B). This interaction is prerequisite for the post-segregational killing mechanism, since its disruption (by mutation) leads to mRNA instability and depletion of the activatable pool of full-length mRNA (Franch & Gerdes, 1996). The 3' element responsible for inhibition of translation and antisense RNA binding was called fbi (fold-back-inhibition). The 5' element that pairs with fbi was denoted tac (translational activation). The tac element is required for activation of translation (Franch & Gerdes, 1996).

In full-length hok mRNA, the SD_mok element is sequestered by an upstream sequence that we denote ucb (upstream complementary box). The ucb element is shown in red in Figure 2. Previously we suggested that pairing between SD_mok and ucb prevents ribosome entry at SD_mok and thereby inhibits translation of mok (and hok; Nielsen & Gerdes, 1995). A nearly perfect repeat of the 9 nt ucb element is located 58 nt downstream of ucb (Figure 2). This element, which pairs with the translation initiation region of hok (TIR_mok) in full-length hok mRNA, was denoted dcb (downstream complementary box). This nomenclature is introduced to illustrate the complex structural rearrangements that occur in hok mRNA (see below). As a consequence of their repetitive nature, the ucb and dcb elements are both complementary to SD_mok (compare Figure 2B and D).

The structure of the antisense RNA target in hok mRNA is yet unknown. However, the genetic algorithm calculations and phylogenetic comparisons presented in the accompanying paper suggest that the exonucleolytic removal of fbi triggers major structural rearrangements at the mRNA 5'end. More precisely, the computer-simulated RNA foldings predict that in truncated mRNA tac pairs with ucb, and SD_mok with dcb. The latter interaction is involved in the formation of an antisense RNA target hairpin (Figure 2D). The target hairpin is topped by a 7 nt loop complementary to the very 5'end of Sok-RNA.

Here, we investigate the biological properties and secondary structures of the full-length and truncated hok mRNAs. We show that the binding of Sok-RNA to full-length hok mRNA is negligible both in vivo and in vitro. Using in vitro toeprint analyses, we show that the mok TIR mRNA is unable to bind ribosomal 30 S subunits, in accordance with the observation that full-length hok mRNA is not translated in vivo. Most importantly, we obtain firm evidence for the postulated structural rearrangements triggered by 3' processing of the full-length mRNA. In the truncated mRNA, tac was found to pair with ucb, and SD_mok with dcb, thus confirming the presence of the tac and target hairpins. A mutational analysis shows that the structure of the target hairpin is crucial for rapid antisense RNA binding and for translation of mok, and that even modest changes in its structure have profound effects on these parameters. We further show that the pairing between SD_mok and dcb is required to maintain truncated hok mRNA in a translatable configuration.

**Results**

**Sok antisense RNA binds to truncated but not to full-length hok mRNA in vivo**

We have previously examined the metabolism of the hok mRNAs in vivo (Gerdes et al., 1990, 1992; Thisted & Gerdes, 1992; Thisted et al., 1994a). Indirect evidence from Northern analyses suggested that Sok-RNA binds to truncated hok mRNA much more efficiently than to the full-length RNA. To test this inference more directly, we constructed a plasmid vector carrying a hok/sok system in which F_hok was precisely replaced by the strong, LacI repressed P_A1/04 promotor (Lanzer & Bujard, 1988). In this system, synthesis of hok mRNA is halted by the removal of isopropyl-β-D-thiogalactopyranoside (IPTG) from the growth-medium. Thus, synthesis
of \textit{hok} mRNA could be blocked without the addition of rifampicin.

The \textit{in vivo} processing pattern of the \textit{hok} mRNAs was analysed by Northern blotting in the presence (\textit{sok}+) or absence (\textit{sok}−) of antisense RNA. In its presence, two \textit{hok} mRNAs, denoted full-length mRNA-1 and mRNA-2, were observed (Figure 3A, first panel). Both mRNAs were quite stable with a half-life of approximately 20 minutes. Previous analyses using rifampicin to inhibit transcription yielded half-lives of the order of 20 to 40 minutes (Gerdes et al., 1990, 1992; Thisted et al., 1994a). \textit{hok}

\begin{figure}[h]
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\caption{Secondary structures of Sok-RNA and \textit{hok} mRNAs. A, Secondary structure of Sok-RNA. B, Secondary structure of full-length \textit{hok} mRNA. C, Configuration of non-refolded truncated \textit{hok} mRNA as predicted by computer-assisted structure calculations. D, Putative truncated, refolded mRNA supported by the structure-probing analyses presented in Figure 4 and the RNase H cleavage pattern shown in Figure 5. Shine-Dalgarno and start codons of the \textit{mok} and \textit{hok} reading frames are shown in blue, and the \textit{ucb} and \textit{dcb} repetitive sequences are shown in red. C74 in \textit{ucb}, marked in green, emphasizes a heterogeneity between the \textit{ucb} and \textit{dcb} elements. Thin numbered lines mark the nucleotides complementary to the three DNA oligonucleotides used in the RNase H cleavage assay. The antisense RNA target that is complementary to the 5'-tail of Sok-RNA is denoted \textit{sokT} (B). The sequence and structure of \textit{super-tac} truncated RNA is shown below the \textit{wt tac/ucb} pairing in D. The non-refolded (C) and refolded (D) configurations are proposed to exist in a dynamic equilibrium.}
\end{figure}
Materials and Methods.

(A) between 32P-labelled Sok-RNA and unlabelled (3H) truncated. The mRNAs contained stop codon mutations in total RNA preparation were taken at the intervals indicated to truncated hok mRNA and thereby confers its rapid decay via RNase III-mediated hydrolysis. Taken together, these results show that Sok-RNA binds full-length hok mRNA at a negligible rate in vivo, whereas the truncated version of hok mRNA is scavenged rapidly.

Figure 3. A, Effect of Sok antisense RNA on the stability and processing pattern of hok mRNAs as shown by Northern blotting. The first panel shows hok mRNAs after arrest of hok transcription in the presence of Sok-RNA, the second panel shows hok mRNAs after arrest of transcription in the absence of Sok-RNA. Cells (CDS30 containing either pKG1540 (sok+) or pKG1541 (sok−)) were grown in LB medium containing 1 mM IPTG to an A650 of ca 0.2, harvested, and resuspended in prewarmed medium without IPTG. Cell samples for total RNA preparation were taken at the intervals indicated. The mRNAs contained stop codon mutations in the hok gene to prevent detrimental hok expression (from Thisted & Gerdes, 1992). B, In vitro binding assay between 32P-labelled Sok-RNA and unlabelled (3H) truncated hok mRNA (first panel) and full-length hok mRNA-1 (second panel). Labelled antisense RNA was added to a tenfold excess of target RNA. For details, see Materials and Methods.

Sok-RNA binds 100-fold more rapidly to truncated than to full-length hok mRNA in vitro

The above results indicate that Sok-RNA binds much more rapidly to truncated than to full-length hok mRNA in vivo. Using a standard in vitro binding assay, we showed previously that full-length hok mRNA bound Sok-RNA with an approximately 30% reduced rate (Thisted et al., 1994b). To resolve this apparent discrepancy, we repeated the in vitro binding assay using a more physiologically correct binding buffer (i.e. 200 mM potassium glutamate was included). The resulting gel-shift experiment is shown in Figure 3B. As seen, Sok-RNA bound readily to the truncated RNA (Figure 3A). However, binding to the full-length RNA was inhibited (Figure 3B). The apparent second-order binding-rate constants (kapp) were estimated to be 3 × 10^5 M⁻¹ s⁻¹ for truncated and less than 3 × 10^3 M⁻¹ s⁻¹ for full-length hok mRNA, corresponding to a more than 100-fold difference in binding-rates (for calculations, see Materials and Methods). This result suggests that the sokT region exists in different configurations in the two RNAs (see Figure 2B and D). Now we can address this important question experimentally.

Secondary structure analyses of full-length, truncated and super-tac truncated hok mRNAs

Mutational analyses, computer calculations and phylogenetic comparisons suggested that translational activation of hok mRNA involves a substantial structural rearrangement at the mRNA 5’-end, and that this rearrangement is triggered by the 3’ processing of full-length hok mRNA (Franch & Gerdes, 1996; Gultyaev et al., 1997, accompanying paper). More specifically, our analyses predicted that in truncated RNA, tac preferentially pairs with ucb thereby leading to formation of the extended tac-stem (see Figure 2D). Although a large internal loop destabilizes the tac/ucb interaction, it contains the possibility of non-canonical GA/AG base-pairing (Turner & Bevilacqua, 1993), which may add to the energy of the tac stem. Most importantly, the 5’ refolding was accompanied by structural rearrangements further downstream in the molecule. Thus, a stem–loop that we coin the antisense mRNA-2 is generated from hok mRNA-1 by an as yet unknown mechanism (Nikolaj Dam Mikkel, unpublished results). The absence of Sok-RNA caused the appearance of a third band corresponding to truncated hok mRNA (Figure 3A, second panel). This RNA is generated by the exonucleolytic removal of 40 nt at the 3’-end of hok mRNA-2 (Gerdes et al., 1990). Comparison of the two panels of Figure 3A yields valuable new information on the in vivo metabolism of hok mRNA: first, the absence of Sok-RNA has no influence on the amounts or the stabilities of the two full-length species. Since antisense RNA binding leads to duplex formation and rapid RNase III cleavage, this observation indicates that Sok-RNA does not bind to hok mRNA-1 or mRNA-2 at a significant rate. Secondly, the absence of Sok-RNA resulted in the accumulation of the truncated mRNA. Since this RNA is absent from the first panel, this observation indicates that Sok-RNA binds avidly to truncated hok mRNA and thereby confers its rapid decay via RNase III-mediated hydrolysis.
RNA target hairpin was formed (Figure 2D and the accompanying paper, Gultyaev et al., 1997). Construction of a “perfect” lac stem by the introduction of the super-tac mutation (see Figure 2D) increased translation of the truncated hok mRNA twofold (Franch & Gerdes, 1996). The super-tac mutation greatly increases the stability of the lac stem. Consequently, the presence of super-tac predictably would lead to refolding of the entire population of hok mRNA molecules present in a pool of in vitro prefolded RNA. This inference is in accordance with the increased translation rate of super-tac truncated RNA.

In order to verify the predicted secondary structure transitions, structure probing analyses were conducted on full-length, truncated and super-tac truncated RNAs (Figure 4). Chemical modification by dimethyl sulphate (modifies N1 of unpaired A > N3 of unpaired C), cleavage by RNase T2 (cleaves 3’ of unpaired nucleotides) and RNase V1 (cleaves paired and stacked nucleotides) were detected by primer extension using the hok1 primer. For convenience, only the regulatory region covering nucleotides 70 to 160 is shown in Figure 4. However, the entire mRNA 5’-ends were also extensively analysed during this work (data not shown).

**Full-length hok mRNA**

In the full-length mRNA, the proposed position of the loop in the ucb/SDmok stem–loop structure
was supported by \( T_2 \) cuts in the region U89-U91 and DMS modification at C92. The DMS modification at A81 supports the internal loop at the top of the stem. Furthermore, \( V_1 \) cuts at 110 to 112 could support the double-stranded bottom stem. Three A-C mismatches (C74-A107, A78-C103 and A82-C100) were predicted in the \( ucb/SD\text{-}mok \) stem. No significant modification of these nucleotides was observed, probably due to the formation of \( A\text{-}C \) reverse wobble pairings (Saenger, 1984). The modifications at C115 and A117, and to a lesser extent at C113 and C114, suggest that this region is at least partially single-stranded. The secondary structure probing of the full-length \( hok \) mRNA is consistent with the structure presented in Figure 2B in which \( sok'T \) is in a double-stranded conformation.

**Wild-type and super-tac truncated \( hok \) mRNAs**

The wild-type and super-tac truncated mRNAs exhibited nearly identical modification/cleavage patterns and comparison with that of the full-length RNA revealed important differences. Cleavage by \( T_2 \) at A78 and A81-A82 in the truncated mRNA suggests the presence of a single-stranded region not present in the full-length transcript. These cuts appeared enhanced in super-tac mRNA. In the SD\text{-}mok\text{-}ucb region significant DMS modifications were observed in super-tac mRNA compared to the weak probing of the wt truncated mRNA, consistent with incomplete 5-end refolding of the latter species. However, DMS and \( T_2 \) probing at C120-U124 confirmed the single-stranded loop of the target stem. In addition, \( V_1 \) cuts at C125, C126 and A127 are consistent with stacking of the CC loop dinucleotide on a stem adenine nucleotide. The super-tac mutation increased the \( T_2 \) cleavages in the loop region (C120 to U124), whereas it reduced cleavage at U117, G118 and A127-A in the stem. Thus, the super-tac mutation seems to stabilize the target hairpin, consistent with incomplete refolding of the wt truncated RNA. This conclusion was corroborated by the \( V_1 \) cuts at C139 in the dcb element. These cuts increase gradually in the order: full-length < truncated < super-tac truncated mRNA consistent with the proposed transition of the dcb/\( TIR\text{-}mok \) interaction in full-length RNA to the SD\text{-}mok/dcb interaction in truncated RNA.

The secondary structure model of truncated \( hok \) mRNA shown in Figure 2D predicts that \( sok'T \) and the \( mok \) TIR are located within the same hairpin. The validity of our secondary structure model of the truncated mRNA was further investigated using an RNase H cleavage assay that tests the propensity of an RNA to hybridize with different DNA oligonucleotides (Zarrinkar & Williamson, 1994). The DNA oligonucleotides used here are indicated in Figure 2.

The cleavage pattern of full-length \( hok \) mRNA is shown in the first panel of Figure 5. Oligo 1 resulted in 6% cleavage only, and oligos 2 and 3 both yielded negligible cleavage. This pattern is consistent with a compact secondary structure, especially in the region of \( sok'T \). In contrast, all three oligonucleotides yielded significant RNase H cleavage of truncated \( hok \) mRNA (second panel). In this case, 38%, 14% and 50% cleavage was observed with oligonucleotides 1, 2 and 3, respectively. The third panel shows the cleavage pattern of super-tac truncated mRNA. Here, oligos 1, 2 and 3 yielded 77%, 19% and 65% cleavage, respectively.

These two latter sets of mapping data are consistent with the formation of the antisense target stem–loop presented in Figure 2D. In addition, oligo 3, which is complementary to \( sok'T \), yielded more than a 100-fold difference in cleavage activity between full-length and truncated mRNA. This pattern is consistent with the difference in antisense RNA binding described above. Oligo 1, which is complementary to SD\text{-}mok, showed an increased cleavage in super-tac truncated mRNA as compared to the wild-type, consistent with the observed twofold increase in translation (Franch & Gerdes, 1996). In addition, the increased cleavage of the super-tac mRNA with all three oligonucleotides corroborates the suggestion that the wild-type truncated mRNA \textit{in vitro} exists in two configurations, a refolded and a non-refolded. This further suggests that the antisense RNA target stem–loop favoured by the \( \text{tac/ucb} \) and SD\text{-}mok/dcb pairings may be in a dynamic equilibrium with the non-refolded structure shown in Figure 2C.
The internal loop of the target hairpin is required for antisense RNA binding.

The loop of the target hairpin in truncated hok mRNA is complementary to the 5' single-stranded end of Sok-RNA (Figure 2A and D). This suggests that the stem–loop structure is crucial for optimal antisense RNA binding. Therefore, we tested the function of the internal loop in antisense RNA binding. Structure-closing mutations yielding a non-interrupted upper stem were introduced at both sides of the target stem (tst1 and tst2). The double tst1,2 mutation was constructed in order to regain the internal loop. The secondary structures of the mutated target RNAs are shown in Figure 6. Antisense binding assays were accomplished as described above using antisense RNAs with 5' ends completely complementary to the mutated hok target RNAs. Typical binding assays are shown in Figure 7, and the relative binding-rates calculated from three independent experiments are given in Figure 6. The observed binding rates for the truncated mRNA carrying tst1 and tst2 were reduced approximately eightfold and 16-fold, respectively. The double mutation (tst1,2) completely restored the binding rate to that of the wild-type RNAs. These results show (i) that interrupted helicity in the target stem is required for optimal antisense RNA binding and (ii) that the postulated secondary structure of the target hairpin is valid.

The internal loop of the target hairpin is required for translation of hok (and mok)

The effect of the tst mutations on hok expression was tested by in vitro translation of truncated mRNAs. Note, that this assay detects translation of hok, not mok. As shown in Figure 8A, the tst1 and
Mutations in the downstream complementary box (dcb) reduce hok expression

In the target hairpin, the SD$_{mok}$ element is proposed to base-pair with the downstream complementary box, dcb (Figures 2D and 6). This sequestration was puzzling, since it is known that secondary structures tend to reduce or even prevent translation (de Smit & van Duin, 1990a,b), and hok mRNA is translated at a significant rate (Figure 8). To investigate this apparent discrepancy, mutations that reduce the pairing to SD$_{mok}$ were introduced into the dcb element (Figure 6). Surprisingly, both dcb mutations resulted in a severe reduction of hok translation (Figure 8A). Thus, the SD$_{mok}$/dcb interaction appears to be required to maintain the wild-type translation rate of hok mRNA.

A straightforward explanation for this unexpected result could be that the SD$_{mok}$/dcb interaction is required for 5' refolding and therefore for translational activation. Hence, weakening of the SD$_{mok}$/dcb interaction would favour the non-refolded ucb/SD$_{mok}$ configuration, thus explaining the effect of the dcb mutations on translation. To test this inference, the super-tac mutation was combined with the dcb1 and dcb2 mutations and the respective RNAs subjected to translation (Figure 8B). The tst1,2 and tst-inv mutations were also combined with super-tac and translation of the respective RNAs included for comparison. The relative translation rates of tst1,2 and tst-inv were not significantly increased by the super-tac refolding mutation. This was expected, since the tst1,2 and tst-inv mutations stabilize the target stem (see Figure 6). In contrast, the super-tac mutation resulted in a three- to sixfold increase in hok translation of RNAs carrying the dcb1 and dcb2 mutations (Figure 6). Thus, the super-tac mutation could, at least partially, reverse the translational defect conferred by the dcb mutations. However, the super-tac dcb mRNA were still translated at a rate significantly lower than that of the wild-type truncated RNA (Figure 8B).

Toeprinting of the mok TIR in full-length and truncated hok mRNAs

To investigate the accessibility of SD$_{mok}$ more directly, we employed the toeprinting technique. The toeprint assay semi-quantitatively monitors the binding affinity between a TIR and the ribosomal 30 S subunit (Hartz et al., 1988). Our toeprint analysis of the mok TIR is shown in Figure 9. In truncated hok mRNA, a weak, but clearly discernible band corresponding to a reverse transcriptase stop at +15 relative to the mok AUG start-codon was detected (lane 4). The position of this toeprint is consistent with a ternary complex formed at the mok AUG codon. We were not able to detect a similar toeprint in full-length hok mRNA (lane 2). This correlates well with the lack of translation of full-length hok mRNA in vivo and in vitro (Gerdes et al., 1990; Thisted et al., 1994a). Furthermore, the weak mok toeprint in truncated hok mRNA suggests that it is translated relatively infrequently, consistent with the pairing of the mok TIR to the dcb element in the target RNA hairpin. Similar results were obtained by the use of another oligonucleotide as primer in the toeprinting assay, indicating that the quantitative aspects described here are independent of the actual experimental setup.

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Figure 8. A and B, SDS-PAGE of in vitro translation reactions of truncated hok mRNA. Relative translation efficiencies are normalized to LacZ(a) and β-lactamase internal standards, and their quantifications are given in Figure 6.

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tst2 mutations abolished hok translation. The tst1,2 mutation partially restored translation (to 9% of that of the wild-type). The tst1,2 mutation increases the ΔG° value of the hairpin by −3.4 kcal/mol because of the G-U base-pairing partially closing the internal loop (Figure 6). Therefore, an additional mutation containing an inverted internal loop sequence was constructed and tested (tst-inv). This mutation reduced the hok translation rate to 17% (Figure 8A). These results are consistent with the notion that the internal loop is responsible for a sufficiently low hairpin stability as to allow loading of ribosomes at the mok TIR.
The accessibility of the mok TIR determines the rate of hok translation

To investigate whether the translatability of hok mRNA and toeprinting at the mok TIR appear to be correlated, we performed toeprinting analyses of mutated hok mRNAs. As seen from lanes 6 and 8 of Figure 9, the presence of the tst1 and tst2 structure-closing mutations completely abolished toeprinting at the mok TIR, consistent with the lack of translation of these RNAs (Figure 6). The tst1,2 and tst-inv mRNAs yielded reduced toeprinting signals (lanes 10 and 12), again consistent with the degree of translation of the mRNAs. The mRNAs containing the dcb1 and dcb2 mutations yielded slightly reduced toeprint signals (lanes 14 and 16) and their translation rates were also clearly reduced (Figure 6). Furthermore, the super-tac mutation that previously was shown to enhance translation of hok by two-fold, yielded a threefold increase in the mok toeprinting signal (lane 18). These results indicate that the strength of the toeprinting signal from the mok TIR and the hok translation rate are correlated. We next asked if the combination of the super-tac mutation and the tst1,2 and tst-inv mutations would enhance the toeprinting signal (lanes 20 and 22, respectively). As seen, the super-tac mutation in these cases did not lead to an increased toeprinting signal. This is consistent with the lack of enhanced hok translation by super-tac in these two cases (Figure 6). Thus, all our observations indicate that within limits, the rate of ribosome loading at the mok TIR determines the rate of hok translation.

The SD\text{mok}/dcb interaction is required for loading of ribosomes at the mok TIR

Even though super-tac to a significant degree reverted the negative effect of the dcb mutations, the double super-tac dcb mutants were not translated as efficiently as the wild-type hok mRNA (Figure 6). One explanation could be that in the double super-tac dcb mutant mRNA mok translation is so intense that it reduces translation of hok. Examples of occluding overlapping translation have been described (Berkhout et al., 1985). In the latter case, toeprinting at the mok TIR yields much more direct information than the hok translation-rate. As seen from Figure 9, the super-tac mutation enhanced the toeprinting signals of both dcb mutant mRNAs more than tenfold (compare lanes 14 and 16 with lanes 24 and 26), and these RNAs clearly yielded the strongest signals of all the mutants. These results are most readily explained by the assumption that super-tac forced refolding of the mRNA into the SD\text{mok}/dcb configuration, combined with the reduced base-pairing between SD\text{mok} and dcb together cause the severe increase in the toeprinting signals of the doubly mutated mRNAs. Taken together, our results show that the antisense RNA hairpin is finely tuned to its two functions, translation and antisense RNA binding, since virtually any change in its structure has detrimental effects.

Discussion

Here, we obtain direct evidence for the refolding model proposed in the accompanying paper.
We predict therein that the 3’ processing of the inactive full-length hok mRNA triggers major structural rearrangements at its 5’-end that activate translation and antisense RNA binding. Accordingly, we show here that the two versions of hok mRNA have highly different properties with respect to translation and antisense RNA binding, and subsequently correlate the biological properties of the molecules with their foldings. Finally, we dissect the antisense RNA target hairpin and show that this structure is required for optimal antisense RNA binding and for proper translation.

Full-length hok mRNA bound Sok-RNA at a negligible rate in vivo (Figure 3A) and in vitro (Figure 3B). These results are consistent with the secondary structure model of the RNA as shown in Figure 2B: the Sok-RNA target region (sokT) is base-paired with the 3’-end of the RNA and is therefore unable to react with the 5’-end of the antisense RNA. Furthermore, the fbi element located at the very 3’-end of the mRNA pairs with the 5’-end element. This pairing prevents lac stem formation and forces the ucb element to pair with SD

In contrast, truncated hok mRNA bound Sok-RNA avidly (Figure 3) and was translated efficiently (Figure 6). Truncated hok mRNA is generated by slow continuous 3’ processing of the full-length mRNA (Gerdes et al., 1990; Thisted et al., 1994a) in both plasmid-carrying and plasmid-free cells. In the former case, the presence of Sok-RNA prevents translation of truncated hok mRNA and mediates its rapid decay via RNase III-mediated hydrolysis (Figure 3A, first panel). Plasmid-free cells, however, do not contain antisense RNA, and truncated hok mRNA therefore accumulates (Figure 3A, second panel). We have shown previously that the 3’ processing leads to Hok protein synthesis and killing of the plasmid-free cells (Gerdes et al., 1990; Thisted et al., 1994a). Thus, the post-segregational killing mechanism is dependent on the formation of truncated hok mRNA.

In the accompanying paper we suggest that the exonucleolytic removal of the hok mRNA 3’-end triggers refolding of the 5’-end with formation of the lac stem and the antisense RNA target hairpin. Figure 2 shows the structural rearrangements proposed to activate both translation and antisense RNA binding. In truncated RNA, lac now prefers to pair with the ucb element (Figure 2D). This interaction favours the formation of the antisense RNA target hairpin. In this hairpin, SD

The pairing of SD
 with dcb was puzzling, since sequestration of SD elements is known to reduce or prevent translation (de Smit & van Duin, 1990a, b, 1994). However, phylogeny and structure-probing data strongly support the postulated interaction. Point mutations in dcb that reduced the SD
/dcb interaction therefore should lead to an increased translation rate. However, the dcb1 and dcb2 mutations both exhibited severely decreased rates of hok translation (eightfold and 16-fold, respectively; see Figure 6). To some extent this result was surprising and indicated that the point mutations perhaps shift the mok TIR into an alternative, non-translatable configuration. One
straight-forward possibility would be that the dcb mutations favour the ucb/SD_{hok} interaction present in the full-length RNA. As shown by toeprinting, this RNA configuration prevents access of ribosomes at SD_{hok} (Figure 9). To test this assumption, we combined the super-tac mutation with the dcb mutations, since super-tac predictably would shift the equilibrium towards the SD_{hok}/dcb interaction and thereby stimulate ribosome-binding at the mok TIR and thus, translation of hok. The super-tac mutation was also combined with the tst1,2, and tst-inv mutations. The super-tac mutation stimulated hok translation of the wild-type mRNA two-fold, consistent with the assumption that super-tac forces refolding of the entire population of molecules (Figure 6). In contrast, super-tac did not stimulate translation of RNAs containing tst1,2 and tst-inv. The latter result shows that translation of these RNAs is refractory to stimulation by super-tac. The target hairpins containing tst1,2 and tst-inv are more energy-rich than the wt hairpin (Figure 6). Thus, the lack of stimulation by super-tac suggests that these RNAs are already fully shifted towards the refolded, active configuration.

In the cases of dcb1 and dcb2, super-tac stimulated translation threefold and sixfold, respectively (i.e. the stimulation was significant). This result is consistent with the proposed structural change conferred by the dcb mutations. However, even though super-tac stimulated hok translation of the dcb mutant RNAs, the translation rates of the doubly mutated RNAs never exceeded that of the wild-type RNA. This was surprising, since super-tac forces formation of the translatable configuration and the dcb mutations reduce the sequestration of SD_{hok}. However, these observations are indirect, since we change the environment of the mok TIR and measure hok translation. This is, of course, a technical caveat.

To gain more direct insight into the status of the mok TIR, we turned to the toeprinting technique (Figure 9). Most importantly, we found that in all but two cases there was a correlation between the in vitro translation rates of hok and the strength of the corresponding SD_{hok} toeprinting signal. In the cases exempt, dcb1 and dcb2, respectively, super-tac severely enhanced the toeprinting signals relative to the translation rates. These results show that the dcb mutations lead, as expected, to a highly accessible SD_{hok} element when combined with super-tac. The relatively slow translation rates from the double super-tac dcb mutant RNAs may be caused by the highly increased mok translation rate, which inhibits hok translation (Berkhout et al., 1985). This inference is consistent with the presence of unexpected Mok protein bands appearing in the translation reactions of these RNAs (data not shown).

The results discussed above suggest that the SD_{hok}/dcb interaction prevents the RNA from folding into the non-translatable configuration. Only when the non-translatable configuration is disfavoured by other structural mutations (i.e. super-tac), the dcb element appears, as expected, to have a negative effect on translation. These assumptions are supported by foldings of the mutated RNAs using the genetic algorithm (not shown). Thus, the toeprinting data explain the somewhat surprising observation that an anti-SD element (dcb) is required to maintain an SD element in a translatable configuration. Our results are still compatible with and actually support the paradigm that secondary structure interactions at SD elements certainly reduce translation (de Smit & van Duin, 1990a, 1994).

In bacteria, exonucleolytic 3' processing is a major pathway of mRNA inactivation and decay (Higgins et al., 1988; Belasco & Higgins, 1988). We believe that the hok and hok-homologous mRNAs represent a unique class of mRNAs that are activated by 3' processing and we know of no other example in which an RNA is activated by 5' refolding triggered by the removal of its 3'-end. However, several other mRNAs contain alternative competing structures involved in translational control. The rpsO mRNA encoding the ribosomal protein S15 and the exoribonuclease PNPase is autoregulated by S15 that binds to the rpsO TIR. The rpsO mRNA leader region exists as a translational-competent pseudoknot structure in equilibrium with a double hairpin structure occluding SD_{mok} (Phillipe et al., 1990). Interestingly, the S15 protein binds and sequesters the pseudoknot structure and thereby inhibits initiation of translation (Phillipe et al., 1993). Also, the leader region of cIII mRNA from phage λ adopts two alternative secondary structures with almost equal energies but different translational capacities (Altuvia et al., 1989). Here, the RNase III protein is believed to trap, without cleavage, the active mRNA configuration (Altuvia et al., 1991).

Many antisense RNAs contain upper-stem mismatches in order to accommodate stable antisense RNA/target interaction (Hjalt & Wagner, 1995; Kittle et al., 1989; Wilson et al., 1997). The structure of the regulatory hairpin in hok mRNA contains a four nucleotide internal loop separated from the seven nucleotide loop by four base-pairs. The stem-closing mutations tst1 and tst2 severely impaired Sok-RNA binding. A similar effect was observed for stem-closing mutations in both CopA of the R1 plasmid replication system (Hjalt & Wagner, 1995) and the RNA-OUT of Tn10 (Kittle et al., 1989). In the hok/sok system, restoring the internal loop by the tst1,2 mutation regained binding kinetics, thus indicating the requirement of helix imperfection for efficient Sok-RNA/target pairing in vitro. Most likely, the initial binding of the 5'-leader of Sok-RNA to the complementary six nucleotides in the target loop creates the first unstable binding intermediate. Our results suggest that subsequent progression to more complete duplex formation requires interrupted helicity to favour intrastrand opening.

In conclusion, we show here that the processing at the hok mRNA 3'-end confers dynamic rearrangements at its 5'-end that mediate activation
of translation and antisense binding. Phylogeny and genetic algorithm calculations indicate that the other hok-homologous mRNAs exhibit similar complex rearrangements (Gultyaev et al., 1997). We believe that this is a unique example of a group of phylogenetically related mRNAs that are activated by structural rearrangements triggered by 3' processing.

Materials and Methods

Enzymes and chemicals

Antibiotics and chemicals was added at the following concentrations: ampicillin, 100 µg/ml; IPTG, 1 mM. All enzymes were purchased from Boehringer Mannheim unless stated otherwise.

Bacterial strains

The Escherichia coli K-12 strain CSH50 [A (lac pro) rpsL] (Miller, 1972) was used in the Northern transfer analysis.

Plasmids

The plasmids used and constructed are as follows: plasmids pKGI540 and pKGI541 are pGEM3 (Promega) derivatives encoding the bla and lacIq genes. The plasmids carry a mutant 450 bp hok/sok system cloned into the EcoRI-BamHI sites of the polylinker. Plasmid pKGI540 is constructed by PCR on a template carrying a stop codon in hok (Thisted et al., 1992) using the oligos (i) and (ii). Oligo (i) is 5'-GGCGCTTGAGGCTTTCTGCCTCATG, italics mark the EcoRI restriction site and the underlined region is complementary to +313 to +356 in the 3'-end of the hok/sok system. Plasmid pKGI541 was constructed accordingly by PCR with the same oligos on a template containing a double mutation in the −10 sequence of the Sok promoter in addition to the hok mutation described above (Thisted & Gerdes, 1992). Calculation of mRNA half-lives was accomplished by evaluation of band intensity using an LKB Ultrascan laser densitometer and the GelScan XL software package (version 2.1) provided by Pharmacia.

Site-directed mutagenesis

The mutation shown in Figure 6 was introduced in the hok/sok system by double PCR as described by Nielsen & Gerdes (1995). PCR was performed using the pBR322 vector carrying the 580 bp wild-type hok/sok system, inserted in the EcoRI- BamHI sites, as template and the pBR322 EcoRI CW and pBR322 BamHI CW external primers. Mutant primers are: ts1, 5'-GGACTAGCAGATTAGGATG-TCATATCATGATGGATGACTAGACATCAGGATG-3' (TF17); ts1, 5'-ATCCGATGTTGAGCAGATCAGCAGATGCTTCC (TF17-5) and 5'-GGCGACTCGTCTAGTGTGACATCAGGATG-3' (TF20a); tst1, 5'-GGCATTACATGTCATGCCCCATCGATGAGAGC-CC (TF20b); tst2, 5'-CACATCACGAGATTCCCTACCCGGCTCCCTAC (TF25a) and 5'-GGCGGTAGGAGGCTTACCGCTACGAGGATAT (TF25b). The ts2 mutation was introduced by double template PCR as described (Franch & Gerdes, 1996) using the primer 5'-ATCCCTATGGCAGCTAGACA TGATGTTGCTCTGTTGG (TF20b); dbc1, 5'-CATCAGATTAGGCTTACCGCCG (TF25a) and 5'-GGCGGTAGGAGGCTTACCGCTACGAGGATAT (TF25b).

Secondary structure probing using DMS, T2 and V1

DMS, T2 and V1 structure probe experiments were conducted according to Thisted et al. (1995), with the following exceptions. The buffer used for DMS modification was as follows: 20 mM HEPES (pH 7.8), 100 mM NH4Cl, 10 mM magnesium acetate, 1 mM DTT and 10% (v/v) glycerol. RNase T1 and V1 cleavage reactions were carried out in TMK-glutamate buffer (20 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 200 mM potassium glutamate).

Secondary structure probing by RNase H cleavage

Uniformly 32P-labelled full-length or truncated hok mRNA (20 fmol) was incubated in TMK-glutamate buffer (see above) supplemented with 1 M DTT, for three minutes at 37°C to equilibrate RNA folding. DNA oligo (10 pmol) and one unit of RNase H (Gibco) were added to give a total reaction volume of 10 µl. The reaction was incubated at 30°C for an additional 30 minutes and stopped by addition of Formamid Dye (FD) and withdrawal to 0°C. In the control reaction the oligo was omitted. Samples were heated at 80°C for three minutes prior to separation on a 5.5% polyacrylamide gel.
in vitro binding reactions between hok mRNA and Sok-RNA

All binding reactions were performed according to Thisted et al. (1994b), except that the hok mRNA and Sok-RNA concentrations were $3 \times 10^{-8}$ M and $3 \times 10^{-9}$ M, respectively. Also, the buffer used was TMK (see above). Band intensity quantification was done by Phosphor Imager and the Imagequant software package (Molecular Dynamics).

In vitro translation

The E. coli coupled transcription/translation system (Zubay, 1973) was purchased from Promega. The translation reactions contained the following in a total volume of 20 μl: 3 pmol of 3H-labelled truncated hok mRNA in 5 μl of TE, 1 μg of pUC19 plasmid (molecular standard), 0.5 mM each of the 20 amino acids minus methionine, 0.2 μM 5'-[32P]methionine (1000 Ci/mmol; NEN), 2 mM ATP, 0.5 mM each of GTP, UTP and CTP, 210 mM potassium glutamate, 20 mM phosphoenolpyruvate, 35 mM Tris-acetate (pH 8.0), 27 mM ammonium acetate, 1 mM cAMP, 20 μg/ml ofolic acid, 100 μg/ml E. coli tRNAs, 9 mM magnesium acetate, 0.8 mM IPTG, 2 mM DTT, 0.4 pmol of 5'-end labelled hok mRNA in 5 μl of FD. An RNA sequence reaction was made to determine the rate of its transcription initiation. J. Mol. Biol. 210, 265–280.

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References


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