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Mechanism of post-segregational killing: translation of Hok, SrnB and Pnd mRNAs of plasmids R1, F and R483 is activated by 3′-end processing

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The gene systems *hok/sok* of R1, *srnB* of F and *pnd* of R483 mediate plasmid maintenance by killing of plasmid-free segregants. Translation of the very stable mRNAs encoding the killer proteins is regulated by small unstable antisense RNAs. The differential decay rates of the inhibitory antisense RNAs and the mRNAs encoding the killer proteins is the basis for the onset of killer mRNA translation in newborn plasmid-free segregants and the killing of these cells. We have suggested previously that this requires that the killer mRNAs occur in two forms. A translationally inactive form was proposed to be converted into a 3′-truncated, translationally active mRNA. In the presence of the antisense RNA, translation from this killer mRNA should be inhibited. In this communication we present *in vivo* and *in vitro* evidence that support this model. The requirement for 3′-processing for killer gene expression is demonstrated. By using *in vitro* techniques it is shown that full-length Hok mRNA is translationally inactive, whereas a 3′-end truncated version of the Hok mRNA is translationally active. *In vitro* secondary structure probing suggests that the 3′-end of the full-length Hok mRNA folds back onto the translational initiation region of the *mok* gene and thereby inhibits translation of the mRNA. By inference we conclude that the Pnd and SrnB mRNAs are regulated by a similar mechanism.

Key words: mRNA processing/plasmid maintenance/post-transcriptional regulation/translational activation

Introduction

A number of antisense RNA regulated gene systems have been described (for a review see Simons and Kleckner, 1988). Normally, the complementary antisense RNA forms a duplex (complete or incomplete) with its target RNA. Such RNA–RNA duplexes are thermodynamically very stable, and inactivation of the target RNA occurs irreversibly. In all cases investigated it has been found that RNase III rapidly cleaves the antisense–target RNA duplex (see e.g. Blomberg *et al.*, 1990; Case *et al.*, 1990; Gerdes *et al.*, 1992). This cleavage leads to a rapid decay of the target RNA, although the significance of this degradation is not clear.

The *hok/sok*, *srnB* and *pnd* systems of plasmids R1, F and R483, respectively, mediate plasmid maintenance by killing plasmid-free segregants (Gerdes *et al.*, 1986a; Nielsen *et al.*, 1991). The systems encode highly stable mRNAs which can be translated into toxic proteins (Hok, SrnB′ and PndA) that kill the cells from within. Translation of the killer mRNAs is repressed by the action of small unstable antisense RNAs that are complementary to the leader regions of the killer mRNAs. The antisense RNAs inhibit translation of reading frames that overlap with the toxin encoding genes (*mok*, *srnB* and *pndC*; see Figure 1). As translation of the killer genes *hok*, *srnB′* and *pndA* is coupled to that of the overlapping reading frames, the antisense RNAs inhibit killer gene expression indirectly (Thisted and Gerdes, 1992).

The differential decay rates of killer gene encoding mRNAs and the inhibitory antisense RNAs, respectively, are the basis for the explanation of the observed onset of killer gene expression in plasmid-free segregants, as well as the induction of killer protein synthesis by the addition of rifampicin (Gerdes *et al.*, 1990a; Nielsen *et al.*, 1991). After plasmid loss or after halt of transcription by the addition of rifampicin (Rif), the antisense RNA decays rapidly. As

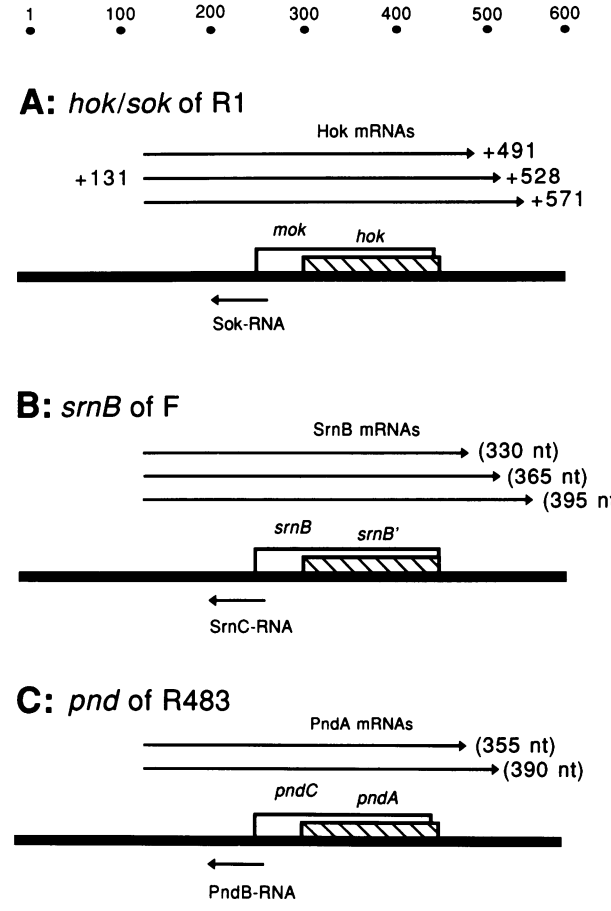

Fig. 1. Genetic organization of the killer gene systems *hok/sok*, *srnB* and *pnd*. Arrows indicate the RNAs encoded by the systems. Cross-hatched boxes symbolize the *hok* and *hok* homologous genes, and open boxes indicate the extension of the *mok* and *mok* homologous reading frames.

de novo mRNA synthesis has ceased, killer gene expression is activated from the remaining pool of stable killer mRNAs.

Initially, how the observed activation of killer mRNA translation in plasmid-free cells (and after rifampicin addition) could be understood seemed an enigma, because (i) in plasmid-carrying cells no *hok* translation should occur, which was thought to imply efficient inhibition by Sok-RNA binding, but (ii) in plasmid-free cells, mRNAs should be reactivatable. Such a reactivation of the Hok mRNA (in a presumed RNA duplex with Sok) appeared paradoxical, because no mechanism has been shown to operate in prokaryotes that can remove an antisense RNA from its target RNA. Thus, we suggested recently that the stable killer mRNAs are largely inactive — both in binding of Sok and in translation of the *hok* gene (Gerdes *et al.*, 1992). Consequently, the bulk of the Hok mRNAs could be a reservoir for the generation of an active mRNA, which in plasmid-containing cells will be subject to Sok-mediated inhibition and in plasmid-free cells (after decay of Sok) will be translated into killer protein.

In this communication we present evidence that substantiates the above predictions. We show that *in vivo* the Hok, SrnB and Pnd mRNAs are specifically cleaved in their 3′-ends, and obtain indirect evidence that this processing leads to activation of their translation. Full-length Hok mRNA was shown to be translationally inactive *in vitro*, whereas the 3′-truncated Hok mRNA was active. Secondary structure mapping of Hok mRNAs indicates secondary structure alterations in the *mok* translational initiation region (TIR), dependent on the presence or absence of the distal 3′-end sequences. This supports a fold-back structure which could be responsible for the low translation activity of the full-length mRNAs.

Because full-length SrnB and Pnd mRNAs also contain potential anti-Shine−Dalgarno (SD) elements in their 3′-ends, we propose a similar regulatory mechanism for all the plasmid maintenance systems belonging to the *hok* gene family (Gerdes *et al.*, 1990b).

In the course of our studies on the regulation of *hok* expression *in vitro*, we observed that Sok/Hok mRNA binding showed some unexpected properties when compared with the behaviour of other, well-characterized antisense RNA systems. The accompanying paper (Thisted *et al.*, 1994) describes an analysis of the secondary structure of Sok-RNA and its binding kinetics to Hok mRNA.

Results

Streptolydigin inhibits accumulation of truncated Hok, SrnB and Pnd mRNAs and expression of the hok, srnB′ and pndA genes

We have analysed previously the *in vivo* RNA patterns of cells harbouring plasmids with the *hok/sok* locus. Figure 2 (left panels) shows the Hok, SrnB and Pnd mRNA patterns *in vivo* before and after the addition of rifampicin. All three systems encode highly stable full-length mRNA species. Furthermore, truncated mRNA species accumulate after Rif addition in all three cases (indicated by asterisks). The truncated mRNAs have the same 5′-ends as the full-length mRNAs but are shortened in the 3′-ends (Gerdes *et al.*, 1990a; Nielsen *et al.*, 1991; A.K.Nielsen, unpublished data).

Expression of the *hok*, *srnB′* and *pndA* killer genes leads to a dramatic change in cell morphology, so-called ghost-cell formation (Gerdes *et al.*, 1986a,b; Nielsen *et al.*, 1991). Thus, expression of the killer genes can be followed by inspection of the cells using phase contrast microscopy. Addition of rifampicin to CSH50 containing *hok/sok*, *srnB* or *pnd*, such as in the above experiment, leads to rapid ghost-cell morphogenesis, indicating that addition of rifampicin induces expression of the killer genes.

Surprisingly, addition of an antibiotic, which is believed to interfere with transcription elongation, had none of these effects. Streptolydigin led to neither the accumulation of the truncated versions of the Hok, SrnB and Pnd killer mRNAs (Figure 2A−C, right panels) nor the formation of ghost-cells. Even though we cannot account for the detailed effect

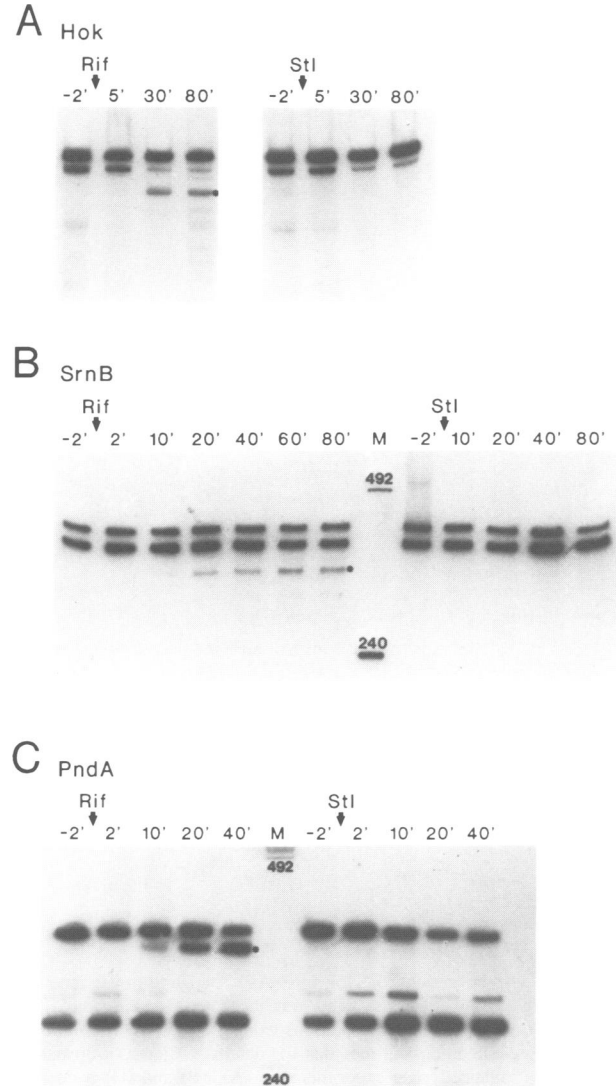

Fig. 2. Processing pattern of the Hok, SrnB and PndA mRNAs after addition of rifampicin or streptolydigin. RNA extracted from (**A**) AS19/pPR633 (pBR-*hok/sok*$^+$), (**B**) AS19/pPT490 (pBR-*srnB*$^+$) and (**C**) AS19/pAN1 (pBR-*pnd*$^+$) before and after addition of rifampicin (Rif; left panels) or streptolydigin (Stl; right panels). The time points of sampling (min) are indicated above each lane. The 3′-end truncated killer mRNAs are indicated by asterisks. The extension of the killer mRNA transcripts revealed by these Northern analyses are depicted in Figure 1. The PndA-specific band with a mobility corresponding to 260 nucleotides is an RNase III cleavage product (A.K.Nielsen, unpublished data). Markers in (B) and (C) were end-labelled DNA fragments of the sizes indicated.

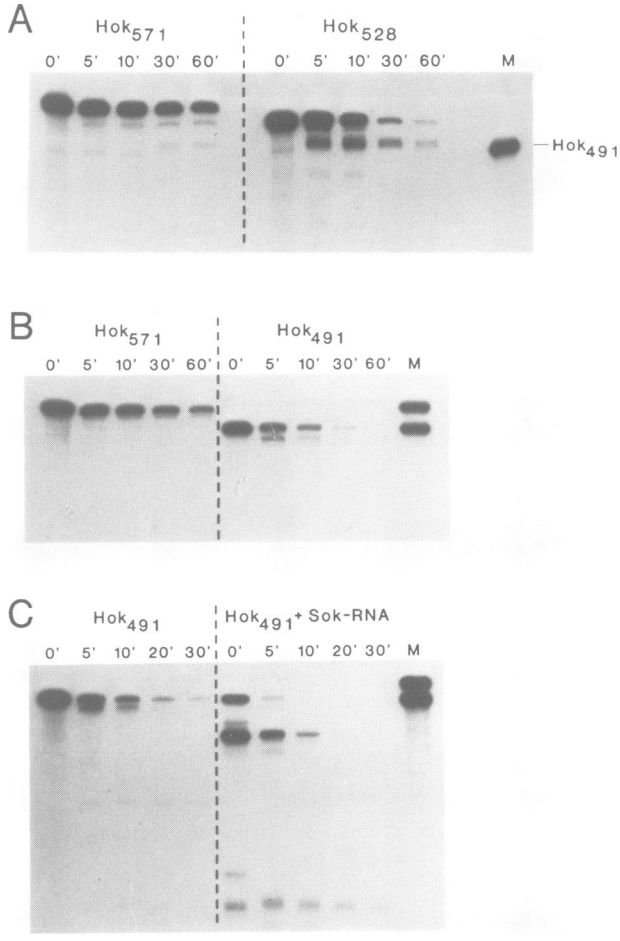

Fig. 3. *In vitro* processing of Hok mRNAs. The same molar amount of uniformly ^{32}P-labelled transcripts was incubated in each assay. The RNAs were incubated under conditions exactly as for the translation reactions (see Materials and methods for details). Aliquots were taken at the times indicated. The experiments in (**A**), (**B**) and (**C**) were run in parallel for direct comparison. (A) Hok_{571} mRNA (left panel) and Hok_{528} mRNA (right panel). The marker was uniformly ^{32}P-labelled Hok_{491} corresponding to truncated Hok mRNA. (B) Relative stabilities of Hok_{571} mRNA (left panel) and Hok_{491} mRNA (right panel). The markers were a mixture of uniformly ^{32}P-labelled Hok_{571} and Hok_{491} mRNA. (C) Hok_{491} mRNA (left panel) and Hok_{491} mRNA preincubated with unlabelled Sok-RNA (right panel). Markers: uniformly ^{32}P-labelled Hok_{571} and Hok_{491} mRNA.

of streptolydigin in this experiment, Figure 2 indicates that the full-length mRNAs present after addition of this drug were not translated, whereas the truncated versions of the mRNAs present after addition of rifampicin were translationally active. These results suggested that the full-length mRNAs encode *cis*-acting elements in their 3′-ends which prevent their translation.

Truncated Hok mRNA is generated by processing of full-length Hok mRNA-2 in vitro

To assess the origin of the truncated Hok mRNA we followed the fate of uniformly ^{32}P-labelled *in vitro* synthesized transcripts corresponding to full-length Hok mRNA-1 or Hok mRNA-2 [denoted Hok_{571} and Hok_{528} mRNA, respectively; the subscripts refer to the coordinates of the 3′-ends of the mRNAs (see Figure 1A)] in a cell extract. As seen in Figure 3A (left panel), Hok_{571} mRNA was very stable when incubated under these conditions, and processed products were not observed. In contrast, Hok_{528} mRNA was considerably more unstable, and a processed product with a size corresponding to the truncated Hok mRNA appeared after 5 min incubation. The cleavage product of Hok_{528} mRNA was further degraded. Accordingly, a transcript corresponding to truncated Hok mRNA (Hok_{491} mRNA) was also degraded when incubated in the *in vitro* system (see Figure 3B, right panel).

The size difference between Hok_{528} mRNA and the processed species seen in Figure 3A (right panel) was due to length heterogeneity at the 3′-end, as shown by oligonucleotide probing of a Northern transfer analysis of the corresponding unlabelled RNA species (data not shown).

These results suggest that the truncated Hok mRNA is primarily generated by 3′-end processing of Hok_{528} mRNA, whereas the longer Hok_{571} mRNA appears to be a poor substrate. This correlates well with the Northern transfer analysis in Figure 2A, where Hok mRNA-2 (corresponding to Hok_{528} mRNA) decreased concomitantly with the appearance of the truncated form. Thus, the truncated mRNA is constitutively generated by 3′-end processing from full-length Hok mRNA-2.

Full-length Hok mRNA is translationally inactive, whereas the truncated Hok mRNA is translationally active in vitro

We translated *in vitro*-generated full-length and truncated Hok mRNAs in a system sustaining coupled transcription/translation (Zubay, 1973; see Materials and methods for details).

As seen in Figure 4A, the *in vitro* translation of an mRNA corresponding to truncated Hok mRNA resulted in one abundant protein (lane 9; Hok_{491}). This was the only specific translation product of this mRNA as all visible minor bands also appear in the control reaction lacking template RNA (lane 1). The band is identified as the Hok protein because translation of truncated Hok mRNA carrying an amber stop codon in the *hok* reading frame (silent in *mok*) does not produce such a band (lane 13; Hok_{491}*hok1*). The molecular weight of the Hok protein was estimated to be ~7.7 kDa from its migration relative to an included set of molecular weight markers. This is slightly larger than the predicted molecular weight of 5.5 kDa. The amount of Hok protein produced was directly proportional to the amount of Hok_{491} mRNA added to the *in vitro* translation system at least up to 4.2 pmol (highest tested amount; data not shown). Surprisingly, no band corresponding to the *mok* gene product (predicted molecular weight of 7.0 kDa) was seen. Labelling with [^{35}S]cysteine instead of [^{35}S]methionine revealed no *mok* product. This suggests that the *mok* product could be very labile in the *in vitro* translation system.

When the same molar amount of an mRNA corresponding to Hok mRNA-1 was included in the translation assay, only a faint band corresponding to the Hok protein was synthesized (lane 3; Hok_{571}), indicating that translation of this mRNA was indeed repressed.

Note that the yield of protein produced from the various mRNAs *in vitro* has to be affected by (i) how well ribosomes can initiate translation on the mRNA and (ii) the functional half-life of the mRNA in the *in vitro* system. Because Hok_{571} mRNA is much more stable than Hok_{491} mRNA in the used *in vitro* system (see Figure 3B; the half-lives of

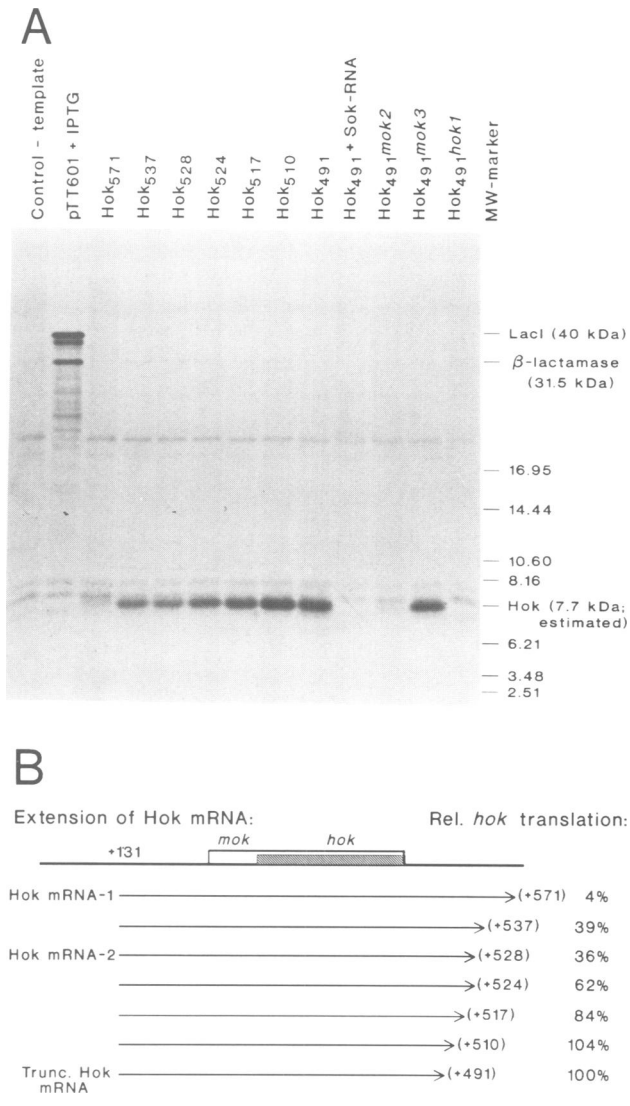

Fig. 4. (**A**) *In vitro* translation of different Hok mRNAs. Autoradiograph of *in vitro* translation reactions labelled with [^{35}S]methionine and run on an SDS−polyacrylamide gel. The mobility of seven unlabelled marker polypeptides visualized by Coomassie staining of the gel, as well as the positions of the two gene products encoded by plasmid pTT601 (LacI and β-lactamase; lane 2), are indicated to the right of the autoradiograph. Lane 1: control translation lacking added template RNA. Lane 2: coupled transcription/translation of an added supercoiled DNA template (plasmid pTT601). Lanes 3−13: translation of 2.4 pmol each of the various *in vitro* synthesized Hok mRNA species (endpoints indicated above the lanes; see text for details). (**B**) Schematic representation of the Hok protein translation yields as a function of 3′-end extension of Hok mRNA. Schematic drawing of the different *in vitro* synthesized Hok mRNAs translated in the experiment shown in (A). The bands corresponding to Hok protein in (A) were quantified by laser densitometric scanning, and the relative intensities compared with the synthesis from Hok_{491} mRNA (100%) was determined.

the transcripts were determined by densitometric scanning of the gel to 40 min for Hok_{571} and 6 min for Hok_{491}, respectively), the difference in the relative translation rates of the two Hok mRNAs is even more pronounced (estimated at >100-fold) than that revealed from the translation experiment shown in Figure 4A.

Since translation activity appears to be strongly repressed by the presence of the RNA region between +491 and +571, we tested a series of successive 3′-truncations for Hok protein synthesis.

We used Hok mRNAs that had different 3′-endpoints, ranging from positions +571 to +491, and tested them for their activities as translation templates. The translation efficiencies of these mRNAs can be compared in Figure 4B. As seen, the amount of Hok protein produced *in vitro* was dependent on the length of the Hok mRNA. However, no sudden change from the inhibited to the uninhibited level of Hok production was found. We consider it likely that this is due to the dependence of translation yield on both the intrinsic translatability of the mRNAs as well as the processing of the mRNAs to the translationally active mRNA during incubation in the *in vitro* system. This issue is considered further in Discussion.

Interestingly, we found a >2-fold increase in expression (from 36 to 84%) when comparing a Hok mRNA with 3′-end at +528 (Hok_{528}, corresponding to Hok mRNA-2) with an mRNA with 3′-end at position +517 (Hok_{517}). Hence, this increase in relative *hok* translation was found when deleting the 11 nucleotides between +517 and +528, suggesting that this region entails at least part of the proposed inhibitory motif.

The secondary structure in the mok TIR is affected by the 3′-end extension of full-length Hok mRNA

The results from the deletion analysis in Figure 4B indicate that sequences between +491 and +528 inhibit *hok* translation. The most probable explanation for the inhibitory action of the 3′-end of full-length Hok mRNA is that sequences in this region interact with the *mok* or *hok* TIR. Presumably, such an interaction could be revealed by changes in secondary structure when probing these regions in full-length and truncated Hok mRNA.

Figure 5 shows the results of a chemical (DMS and CMCT) and enzymatic (T_1, T_2 and V_1) probing experiment of full-length (Hok_{571}) and truncated Hok mRNA (Hok_{491}) performed in parallel. The only significant changes in modification/cleavage pattern occurred in a region of about 30 nt encompassing the middle of the *mok* SD as well as the *mok* AUG start codon. The functionally most important difference when comparing Hok_{571} with Hok_{491} is that in Hok_{491} the first G in the *mok* SD sequence (AC*G*AGG) and the G residue in the *mok* AUG codon clearly become accessible. In all other regions of the two Hok mRNAs investigated [the primer extension analysis covered the region from the 5′-end (+131) to +471 using three different primers], the presence or absence of the 3′-segment of the RNAs did not affect the modification or cleavage patterns. A more comprehensive secondary structure analysis of the Hok mRNA will be published elsewhere.

Hence, the presence of sequences in the 3′-end of Hok_{571} mRNA affects the structure in the upstream part of the molecule encompassing the *mok* TIR. This suggests that the difference in secondary structure observed (Figure 5) could be responsible for the difference in translatability of full-length and truncated Hok mRNAs, respectively. The proposed inhibitory element located in the 3′-end of the full-length Hok mRNA was termed *fbi* [**f**old-**b**ack **i**nhibition; a term 'invented' by R.W.Simons for the IS*10* transposase mRNA (personal communication)].

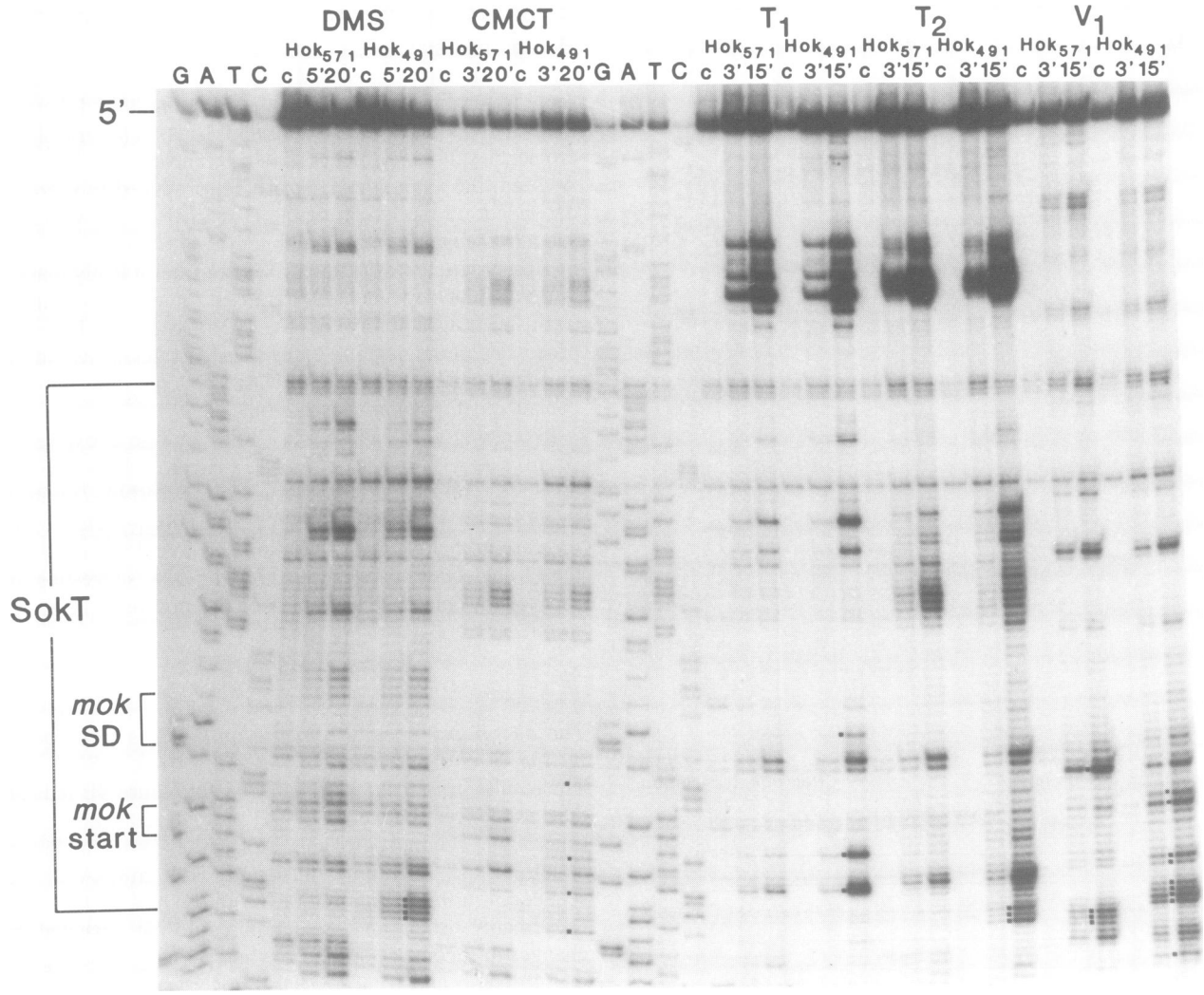

Fig. 5. Changes in secondary structure of Hok mRNA dependent on the presence or absence of the 491–571 region. Figure 5 shows an autoradiograph that identifies sites of chemical modifications with DMS and CMCT, as well as sites of enzymatic cleavages with RNases T_1, T_2 and V_1 in Hok_{571} and Hok_{491} mRNA, as detected by primer extension. Lanes marked 'c' indicate controls performed without modifying reagents or enzymes. G, A, T and C represent dideoxy sequencing reactions generated by primer extension of untreated RNA. The positions of the *mok* SD and start codon, as well as the target for the antisense RNA, SokT, are indicated. Positions of enhanced modification or cleavage of one mRNA species relative to the other are indicated by dots.

A stop codon mutation in mok upstream of the hok TIR abolishes hok translation in vitro

Stop codon mutations introduced in *mok* upstream but not downstream of the *hok* SD region abolish *hok* expression as well as the plasmid stabilizing property of the system. This was taken as an indication that translation of *mok* through a region encompassing the *hok* SD region is a prerequisite for efficient expression of *hok in vivo* (Thisted and Gerdes, 1992). We tested whether the *mok* dependence of *hok* translation was faithfully reproduced in the *in vitro* system used.

Translation of a truncated Hok mRNA containing an upstream stop codon mutation in *mok* (the *mok2* mutation located in the third codon of *mok*) expressed very low amounts of Hok protein (Figure 4A, lane 11; Hok_{491}*mok2*) compared with the amount of Hok synthesized from an equimolar amount of wt truncated Hok mRNA (lane 9; Hok_{491}). In contrast, the yield of Hok protein synthesized from a truncated Hok mRNA carrying a stop codon mutation in *mok* downstream of the *hok* TIR (*mok3* in the 25th codon of *mok* which is silent in the *hok* reading frame) did not affect translation of *hok* (lane 12; Hok_{491}*mok3*). This observation lends support to the previously proposed obligatory translational coupling between *mok* and *hok*.

Addition of Sok-RNA abolishes hok expression in vitro

Several lines of evidence have indicated that Sok-RNA inhibits *hok* expression *in vivo* (see Thisted and Gerdes, 1992). We tested whether Sok-RNA was able to repress *hok* translation *in vitro*. This experiment is important because in some systems, target RNA activity is inhibited by the antisense RNAs while they are being synthesized (see e.g. Eguchi *et al.*, 1991), whereas in the *in vitro* system employed here, both mRNAs and Sok-RNA are added pre-synthesized.

As seen in Figure 4A (lane 10), the preincubation of the Hok_{491} mRNA with a molar excess of Sok-RNA under conditions sufficient for RNA duplex formation (Thisted *et al.*, 1994) essentially abolishes Hok protein production

compared with the one seen in the absence of Sok-RNA (Figure 4A; compare lane 9 with lane 10).

In addition to an expected inhibition of *hok* translation via *mok* translation through the binding of Sok at the *mok* TIR, we also expect an additional effect on the functional half-life of Hok mRNA *in vitro*. This is due to the RNase III-dependent degradation of the Sok−Hok mRNA duplex (Figure 3C; Gerdes *et al.*, 1992). Hence, the inhibitory effect of Sok-RNA on Hok protein production from Hok_{491} mRNA in the *in vitro* system is most likely the result of a combination of translational inhibition and increased turnover of the mRNA.

In conclusion, both the dependence of *hok* translation on *mok* translation and its effective inhibition by the antisense RNA indicate that the results from the *in vitro* system reflect the regulatory properties of the *hok/sok* system.

Discussion

In this communication we describe the use of an *in vitro* system by which we have analysed processing, translation and regulatory properties of the transcripts encoded by the *hok/sok* system.

Recent data obtained *in vivo* for the *hok/sok* system indicated that the accumulation of a 3′-end truncated Hok mRNA species correlated with efficient expression of the Hok protein (Gerdes *et al.*, 1990a). The *in vivo* data presented in this communication further supported the significance of the truncated mRNAs previously observed in the *hok/sok*, *srnB* and *pnd* systems. Addition of rifampicin led to slow accumulation of the truncated mRNAs in all three cases and a concomitant induction of killer gene expression. Addition of streptolydigin led to neither the accumulation of the truncated mRNAs nor the induction of killer gene expression. Although the immediate effect of streptolydigin in this system is not understood, we can conclude that the result supports the correlation between killer mRNA activity and the occurrence of the truncated mRNA variant, whereas the full-length mRNAs by inference are translationally inactive.

Northern analysis of Hok mRNAs produced *in vivo* showed that two species were present before addition of rifampicin, the full-length Hok mRNA-1 and -2. After addition of rifampicin, the Hok mRNA-1 appeared stable whereas Hok mRNA-2 decayed slowly, concomitantly with the emergence of the truncated Hok mRNA. Messenger RNAs corresponding to Hok mRNA-1 and -2 (Hok_{571} and Hok_{528} mRNAs) were synthesized *in vitro* and exposed to an *Escherichia coli* S30 protein extract. As seen in Figure 3A, Hok_{571} mRNA was very stable, whereas Hok_{528} mRNA was slowly processed to an mRNA with the same mobility as truncated Hok mRNA. Using Northern analysis, we showed that Hok_{528} mRNA was processed *in vitro* in its 3′-end (data not shown). Thus, the processing patterns of the Hok mRNA-1 and -2 are very similar *in vivo* and *in vitro*. Hok mRNA-1 is surprisingly stable *in vivo* and *in vitro*. As indicated in Figure 6A, a highly stable stem−loop structure is potentially present in the 3′-end of full-length Hok mRNA-1, and this structure could act as a barrier for 3′−5′ exonucleolytic degradation, thus protecting Hok mRNA-1 from processing and degradation.

The exact nature of the enzymatic reaction (endo- or exonucleolytic) resulting in the 3′-end processing of the Hok,

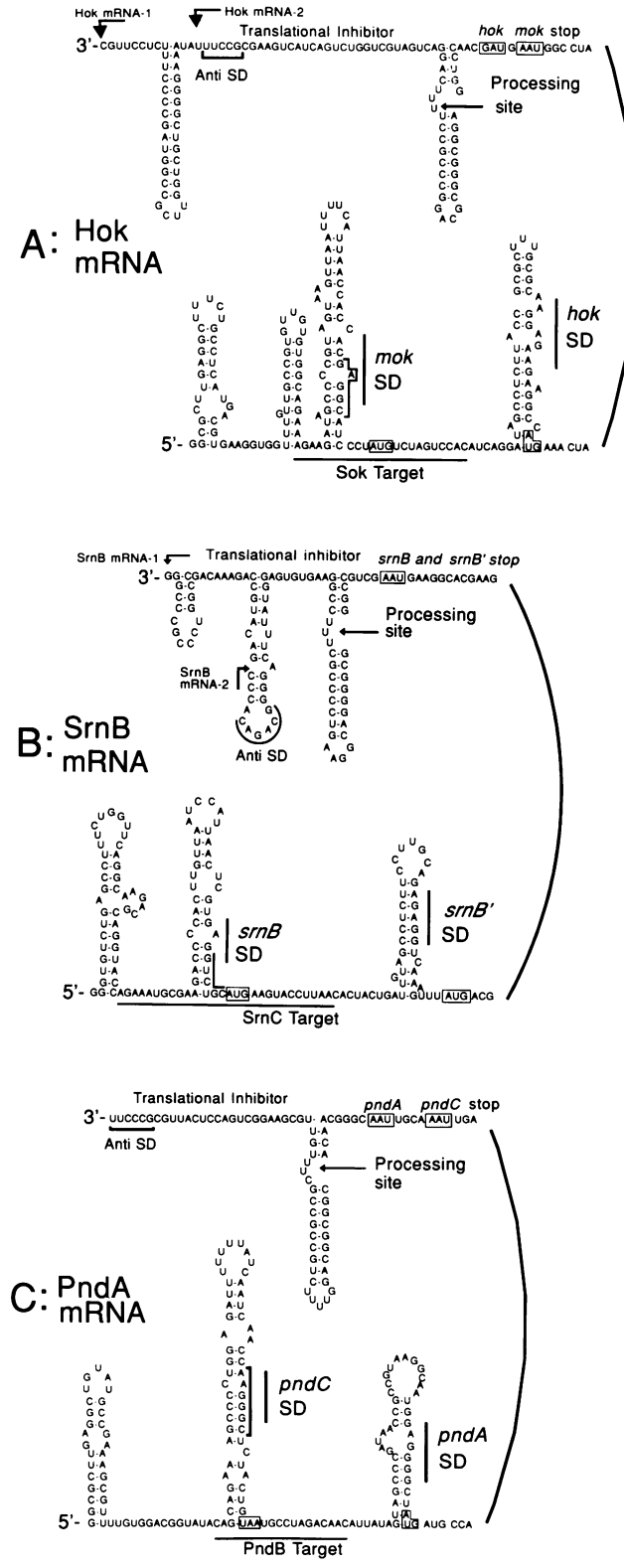

Fig. 6. Proposed local secondary structures and proposed long-range interaction of the Hok, SrnB and Pnd mRNAs. The proposed secondary structures of the Hok, SrnB and Pnd mRNAs were determined by visual inspection of the sequences and by computer-assisted RNA secondary structure analysis (Zuker and Stiegler, 1981). SD, Shine-Dalgarno sequences. Potential anti-SD motifs are indicated. The nucleotides complementary to the anti-SD motifs are bracketed. Start and stop codons are boxed. The target regions of the antisense RNAs are underlined.

SrnB and Pnd mRNAs is unknown at present. The sequence and proposed structure at the positions of cleavage in the three mRNAs are similar, but have no obvious match with any known endonuclease cleavage sites (Figure 6). Streptolydigin inhibited the accumulation of the truncated Hok, SrnB and Pnd mRNAs *in vivo*. Thus, the truncated mRNAs seem to be generated by the same type of reaction. Because streptolydigin is a specific inhibitor of the elongation reaction of *E.coli* RNA polymerase (Schleif, 1969; Severinov *et al.*, 1993), our results suggest that processing may be coupled to transcription or transcription termination. Alternatively, streptolydigin could inhibit a cellular RNase activity involved in killer mRNA processing. Curiously, streptolydigin did not impede the processing of pre-synthesized Hok_{528} transcripts added to the *in vitro* system (data not shown). The reason for this discrepancy between the *in vivo* and *in vitro* results is unknown and is currently under investigation.

Using a cell-free system, we show that Hok_{571} mRNA (corresponding to Hok mRNA-1) is translationally inactive, whereas Hok_{491} mRNA (corresponding to truncated Hok mRNA) is translated efficiently *in vitro* (a >25-fold increase in translational efficiency). Thus, a stretch of nucleotides within the 3′-terminal region of Hok_{571} mRNA inhibits *hok* translation. The inhibitory motif was mapped by translation of a series of Hok mRNAs with progressively shortened 3′-ends. An inverse correlation between the length of mRNA and Hok protein synthesis was obtained (Figure 4B). The total amount of Hok protein produced in the *in vitro* translation reactions is affected by several parameters. For instance, Hok_{528} mRNA was efficiently processed to the truncated Hok mRNA in the *in vitro* system (see Figure 3A; right panel). The same processing pattern and kinetics were observed for Hok_{537} mRNA (data not shown). Therefore, the total amount of Hok obtained from translation of Hok_{528} and Hok_{537} mRNA is most likely a result of the low intrinsic translation of these mRNAs themselves, and the translation from the truncated Hok mRNA resulting from the processing of the mRNAs during incubation in the extract, i.e. Hok_{491} mRNA is translated efficiently, whereas the Hok_{571} and Hok_{528} mRNAs are not. Based on this reasoning and the deletion analysis shown in Figure 4B, we inferred the presence of an inhibitory element in the region +491 to +528.

The mapping of the inhibitory element *in vitro* is consistent with the data obtained *in vivo*: Hok mRNA-1 and -2 are both present in significant amounts in steady-state (i.e. before Rif addition; see Figure 2A). These mRNAs are not translated because this would lead to cell killing, neither are they in complex with Sok-RNA because this would lead to rapid RNase III cleavage (Gerdes *et al.*, 1992). Therefore, the *in vivo* data support the presence of an inhibitory element located in the Hok mRNA between +491 (3′-end of truncated mRNA) and +528 (3′-end of Hok mRNA-2).

Two lines of evidence suggest intramolecular long-range RNA−RNA interaction as the basis for the absence of translational activity of the full-length Hok mRNA. First, the presence of the 3′-sequences prevent *hok* translation, and because *mok* translation is a prerequisite for *hok* translation, an interaction between the *mok* TIR and the 3′-end of the mRNA appears likely. Secondly, the secondary structure analysis presented in Figure 5 shows that the RNA folding is changed locally in the *mok* TIR. In particular, these changes are consistent with partial sequestration of this region by the 491−528 region of the full-length mRNA. We call the inhibitory motif *fbi*. Figure 7 shows two alternative

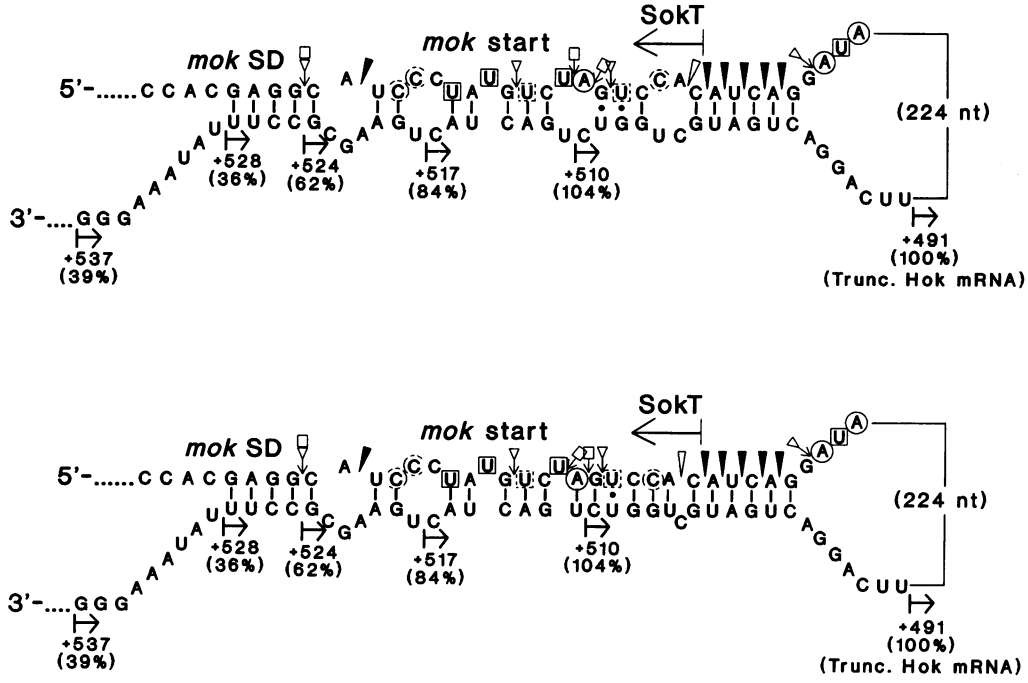

Fig. 7. Proposed fold-back inhibitory structure (*fbi*) in full-length Hok mRNA. Graphical representation of two alternative intramolecular pairings between the *fbi* motif in the 3′-end of full-length Hok mRNA and the *mok* TIR are shown. The *mok* SD, *mok* start codon and the 3′-border of the SokT region are indicated. The 3′-ends of the various *in vitro* synthesized Hok mRNAs together with their relative translational efficiency (taken from Figure 4B) are indicated. Sites of DMS (○) and CMCT (□) modification, as well as cleavage by nuclease T_1 (▽), T_2 (▽) and V_1 (▽) of Hok_{571} mRNA as obtained in several experiments similar to the one shown in Figure 5, are superimposed on the structural models. Hatched/open symbols represent weak modification or cleavage; closed/filled symbols indicate stronger modification or cleavage, respectively.

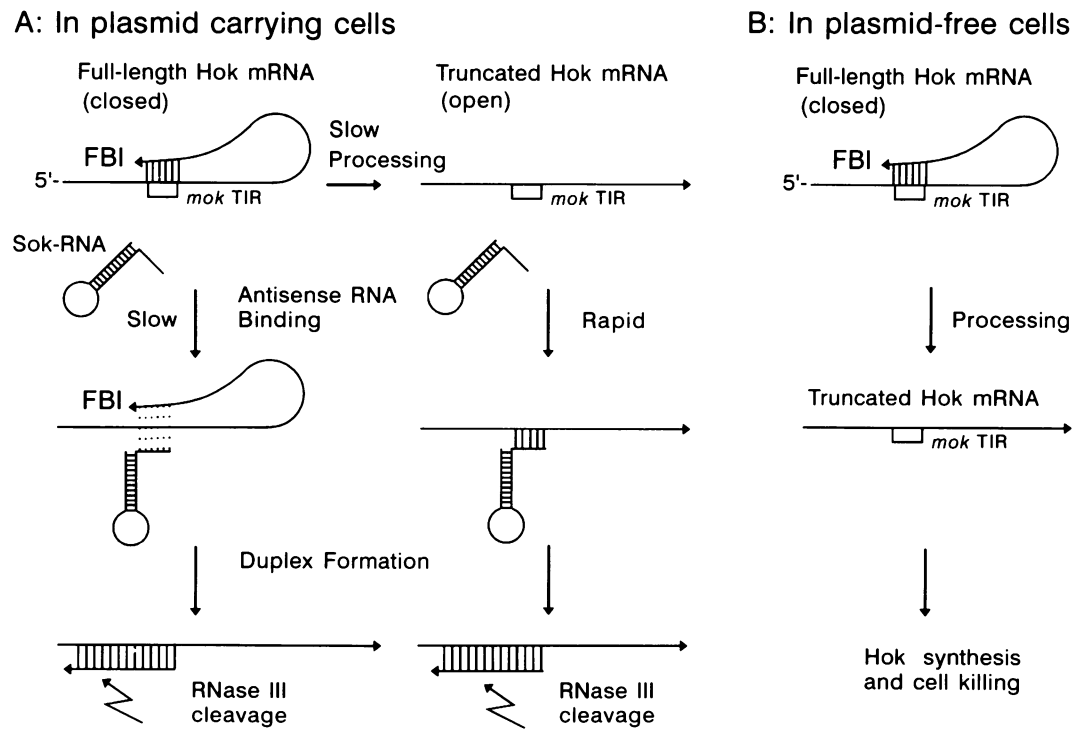

Fig. 8. Schematic model explaining activation of translation of Hok mRNA in plasmid-free segregants. See text for details.

models for this long-range interaction, both of which are largely consistent with the secondary structure mapping analysis performed on Hok_{571} mRNA. By sequence comparison we were also able to identify elements within the corresponding mRNAs of the SrnB and Pnd systems that could carry out analogous functions (Figure 6). Secondary structure information on these two systems is not yet available, but the demonstrated similarities with respect to the truncated/full-length killer mRNA patterns, as well as the essentially identical functions of these systems (discussed in Gerdes *et al.*, 1990b), suggest that killer gene expression occurs by the mechanism outlined above.

The model proposed for regulation of post-segregational killing is depicted in Figure 8. The feature that makes this regulatory system unique is that its function resembles a 'molecular memory'. The *hok* gene product is only expressed in cells devoid of the gene that encodes it. The genetic and biochemical characterization of the *fbi* elements presented in this paper resolves the problem of how inactive, but activatable Hok mRNAs can be inherited by plasmid-free daughter cells. The present model postulates that translation of Hok mRNAs requires the removal, by 3′-processing, of an inhibitory stretch of nucleotides. In plasmid-carrying cells, *hok* translation from the truncated mRNAs can still be inhibited by binding of Sok-RNA to the *mok* TIR. In plasmid-free cells, the rapid decay of Sok eventually leads to uninhibited *hok* expression from the remaining activated Hok mRNAs. Thus, an important element of this model is the presence of a reservoir of very stable, but translationally inert, full-length Hok mRNAs. From the results presented here and in Thisted *et al.* (1994), it appears that the *fbi* element can provide the dual functions required. On the one hand, the long-range *fbi*–*mok* TIR interaction prevents translation of *mok* (and thereby *hok*) which otherwise would result in cell killing, and on the other hand it prevents Sok-RNA from binding to the same TIR region (see also Thisted *et al.*, 1994). Rapid binding of Sok to the full-length mRNAs would be detrimental for functioning of this system, because the Sok–Hok mRNA duplex is rapidly degraded in an RNase III-dependent fashion (Gerdes *et al.*, 1992). Consequently, the Hok mRNA pool would be turned over quickly and would not be inherited stably to plasmid-free daughter cells. The slow constitutive activation that manifests itself by the appearance of the truncated mRNAs lacking *fbi* can then still be counteracted by Sok if the antisense RNA is present (plasmid-containing cells), but becomes irreversible if plasmids are absent.

Several details of the proposed model are still awaiting clarifying experiments. Thus, the nature of the translational coupling between *mok* and *hok* is not understood. From secondary structure predictions and limited secondary structure analysis it is likely that the sequestering of the *hok* TIR in a stable stem–loop structure prevents the independent translation of this reading frame (see Figure 6A). Similar cases of translational coupling have been reported and inhibitory secondary RNA structures have been implicated (for reviews see Gold, 1988; de Smit and van Duin, 1990).

The energetics of the formation of the proposed long-range secondary structure is not known, nor is its stability. In several systems, long-range RNA–RNA interactions have been proposed to affect translational initiation by sequestering the translational initiation region of certain genes into structures inaccessible for the ribosomes. The examples include the *gnd* gene of *E.coli* (Carter-Muenchau and Wolf, 1989), the phage T7 *1.2* gene (Saito and Richardson, 1981), *tnp* of IS*10*R (Kleckner, 1989; R.W.Simons, personal communication), the *E.coli* gene *rplJ* (Petersen, 1989) and the *21k* gene in the *trmD* operon of *E.coli* (Wikström *et al.*, 1992).

An activation of translation via endonucleolytic cleavage

of an mRNA eliminating inhibitory secondary structures has been reported for some systems. Two examples come from the transcripts encoded by the early region of bacteriophage T7 (Studier *et al.*, 1979; Saito and Richardson, 1981), and one from activation of lambda *N* gene expression (Kameyama *et al.*, 1991). In all of these cases, RNase III processing is responsible for removal of the inhibitory secondary structures.

In conclusion, we propose that a sequence element in the 3′-region of full-length Hok mRNA (*fbi*), through formation of an intramolecular long-range RNA−RNA interaction, is involved in inhibiting the synthesis of the Hok killer protein. We also show, in Thisted *et al.* (1994), that this fold-back structure can inhibit Sok-RNA−Hok mRNA duplex formation. Both functions of the *fbi* element are required to plausibly explain the biology of the killer phenotype.

Materials and methods

Media, enzymes and chemicals

Rifampicin (Ciba-Geigy), 100 μg/ml, and Streptolydigin (Up-John Company), 100 μg/ml, were used. Enzymes were purchased from Boehringer unless otherwise stated.

Bacterial strains

For Northern transfer analysis, the rifampicin and streptolydigin permeable *E.coli* B strain AS19 (Sekiguchi and Iida, 1967) was used. For all other purposes, the host was the *E.coli* K-12 strain CSH50 [Δ(*lac pro*) *rpsL*; Miller, 1972].

Plasmids

pPR633 (Rasmussen *et al.*, 1987) carries the 580 bp wt *hok/sok* system cloned in the *Eco*RI−*Bam*HI sites of pBR322. pTT625, pTT626 and pTT640 are mutant derivatives of pPR633 carrying the *mok3*, *mok2* and *hok1* mutation, respectively (Thisted and Gerdes, 1992). pGEM342 carries the downstream *hok/sok* *Sau*3A (+342)−*Eco*RI (+580) fragment cloned in pGEM-blue (Gerdes *et al.*, 1990a). pTT601 was designed as a Mok overproducing plasmid. A linker carrying the *cro* SD was cloned in front of the *mok* reading frame in a *hok1* mutant (silent in *mok* but preventing lethal *hok* expression). The fragment containing *cro* SD and the entire *mok* reading frame was inserted in the *Bam*HI−*Eco*RI sites behind the p_{tac} promoter in pTTQ19 (*lacI*^{q} *bla*$^+$; Stark, 1987) (this work). pAN1 (pBR322-*pnd*$^+$), pAN7 (carrying downstream +330 to +756 *pnd* fragment inserted into pGEM4), pPT490 (pBR322-*srnB*$^+$) and pPT4 (carrying downstream +196 to +398 *srnB* fragment inserted into pGEM4) have all been described in Nielsen *et al.* (1991).

Preparation of Hok mRNAs and Sok-RNA

Hok transcripts of different length (with varying 3′-ends) were synthesized *in vitro* using T7 RNA polymerase and DNA templates generated by PCR. The T7-1 oligo, complementary to +131 to +157 in the *hok/sok* system and containing the T7 promoter sequence, was used in combination with one of the other oligos listed below, specifying the length of the DNA fragment and hence the 3′-end of the resulting run-off transcript as indicated: T7-1, 5′-CGGGATCCTGTAATACGACTCACTATAGGGCGCTTGAGGCTTTCTGCCTCATG-3′ (region of complementarity to *hok/sok* is underlined); T7-2, 5′-GCAAGGAGAAAGGGGCTAC-3′ [3′-end at +571 (= Hok mRNA-1)]; T7-3N, 5′-AAGGCGGGCCTGCGCCCGCCTCCAGG-3′ [3′-end at +491 (= truncated Hok mRNA)]; T7-4, 5′-AAGGCGCTTCAGTAGTCAG-3′ [3′-end at +528 (= Hok mRNA-2)]; T7-5, 5′-GACCAGCATCAGTCCTGAA-3′ (3′-end at +510); T7-6, 5′-GTAGTCAGACCAGCATCAG-3′ (3′-end at +517); T7-7, 5′-CCCTTTATAAAGGCGCTTCAG-3′ (3′-end at +537); and N4, 5′-CGCTTCAGTAGTCAGACCA-3′ (3′-end at +524).

The DNA fragment amplified by PCR was recovered from an agarose gel, phenol and $CHCl_3$ extracted, precipitated and resuspended in H_2O, and finally transcribed *in vitro* with T7 RNA polymerase in a 100 μl transcription mixture of the following composition: 40 mM Tris−HCl pH 7.5, 2 mM spermidine, 10 mM NaCl, 6 mM $MgCl_2$, 11.5 μM [^{3}H]CTP (NEN), 17 μM CTP, 100 μM each of ATP, GTP and UTP, 10 mM DTT, 100 U RNasin (Promega), 80 U T7 RNA polymerase (Promega) and ~1−2 μg purified PCR template. For [^{32}P]CTP-labelling, [^{3}H]CTP was replaced with 1.25 μM [^{32}P]CTP (NEN) (in the text ^{3}H-labelled transcripts are referred to as unlabelled). The transcription reaction was performed at 38°C for 2 h. For large scale T7 transcription we employed the following overnight transcription procedure: a reaction volume of 100 μl contained 40 mM Tris pH 7.5, 2 mM spermidine, 10 mM NaCl, 24 mM $MgCl_2$, 7.8 μM [^{3}H]CTP, 4.0 mM each of ATP, GTP, CTP and UTP, 10 mM DTT, 100 U RNasin (Promega), 37.5 U T7 RNA polymerase (Promega) and ~3−5 μg DNA template. The transcription reaction was performed overnight at 37°C.

In vitro transcription with *E.coli* RNA polymerase was performed essentially as described by Persson *et al.* (1988). For [^{32}P]CTP-labelling, the transcription reaction contained the following in a volume of 100 μl: 40 mM Tris−HCl pH 7.9, 150 mM KCl, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA, 1 mM DTT, 1.0 μM [^{32}P]CTP, 40 μM CTP, 200 μM each of ATP, GTP and UTP, 40 U RNasin, 3 U *E.coli* RNA polymerase and 5−10 μg supercoiled pGEM154 (*sok*$^+$) template DNA purified by banding in CsCl gradients. For synthesis of ^{3}H-labelled Sok-RNA, [^{32}P]CTP was replaced with 7.8 μM [^{3}H]CTP. The transcription reaction was allowed to proceed for 1.5 h at 37°C.

Transcript recovery was performed essentially as described in Persson *et al.* (1988). Transcripts were electrophoresed with corresponding ^{32}P-labelled markers on 5.5 (Hok mRNAs) or 7% (Sok-RNA) polyacrylamide 6 M urea gels. The transcripts were eluted from the gel by soaking the crushed gel slices in 500 μl elution buffer (0.1 M NaOAc pH 5.7, 10 mM Na_2EDTA, 0.5% SDS) containing 6 μg carrier tRNA at 20°C for 50 min. Gel debris was removed by centrifugation. Transcripts were phenol and chloroform extracted, precipitated twice with ethanol, washed and dried.

Upon gel purification, the transcripts were dissolved in TE buffer (10 mM Tris−HCl pH 7.9, 1 mM Na_2EDTA). The presence of the carrier tRNA added during recovery did not affect the *in vitro* translational efficiency of the transcripts (data not shown). Concentration of [^{3}H]CTP-labelled transcripts was determined by liquid scintillation counting.

Total RNA preparation and Northern transfer analysis

Preparation of total RNA from *E.coli* and Northern transfer analysis was performed as described earlier (Thisted and Gerdes, 1992). The ^{32}P-labelled RNA probes used were generated using T7 RNA polymerase and the pGEM-based plasmids pGEM342 (Hok probe), pAN7 (Pnd probe) and pPT4 (SrnB probe) as described previously (Nielsen *et al.*, 1991; Thisted and Gerdes, 1992).

Test of transcript stability in E.coli S30 extracts

The uniformly ^{32}P-labelled transcripts were incubated at 37°C in a mixture sustaining coupled transcription/translation containing an *E.coli* S30 protein extract as described below. Hence, the conditions were exactly the same as for the S30 translation experiments described below except that unlabelled methionine was exchanged for [^{35}S]Met. Samples were withdrawn and phenol extracted immediately, run on 5.5% acrylamide gels, which were dried down and autoradiographed.

In vitro translations

The *E.coli* coupled transcription/translation system (Zubay, 1973) was purchased from Promega. The translation reactions contained the following in a total volume of 25 μl: 2.4 pmol ^{3}H-labelled Hok mRNA in 7 μl TE (for the coupled transcription−translation control, 1 μg pTT601 supercoiled DNA template), 0.5 mM each of the 20 amino acids minus methionine, 0.2 μM L-[^{35}S]methionine (1000 Ci/mmol; NEN), 2 mM ATP, 0.5 mM each of GTP, UTP and CTP, 210 mM potassium glutamate, 20 mM PEP, 35 mM Tris−OAc pH 8.0, 27 mM NH_4OAc, 1 mM cAMP, 20 μg/ml folinic acid, 100 μg/ml *E.coli* tRNAs, 9 mM $Mg(OAc)_2$, 0.8 mM IPTG, 2 mM DTT, 35 mg/ml PEG 8000 and 7.5 μl *E.coli* S30 protein extract.

The translation reaction was allowed to proceed at 37°C for 35 min. For the experiment with Sok-RNA inhibition, 1.5 times molar excess of Sok-RNA was included and allowed to bind for 25 min at 37°C before addition of the S30 protein extract. The samples were precipitated with four volumes of acetone at 0°C, dried and redissolved in 2 × SDS sample buffer [per 10 ml: 2.0 ml glycerol, 2.0 ml 10% SDS, 0.25 mg bromophenol blue, 2.5 ml (0.5 M Tris−HCl pH 6.8, 0.4% SDS), 0.5 ml β-mercaptoethanol] and denatured at 100°C for 5 min prior to loading on SDS gels.

As a control for the amount of mRNA translated, dilutions of RNA samples were tested in an *in vitro* binding assay in which a molar excess of ^{32}P-labelled antisense RNA was boiled in on its target (see Thisted *et al.*, 1994, for details).

SDS−PAGE

For optimal resolution of proteins in the low molecular weight range (5−20 kDa), the discontinuous tricine−SDS−PAGE system described by Schägger

and von Jagow (1987) was used. The stacking gel contained 4% acrylamide and separation gel 16% acrylamide. Electrophoresis was carried out at 30 V in the stacking gel, and when the sample had reached the separation gel the current was increased to a constant 25 mA through the remainder of the run (total run time, 20 h). The gel was then fixed for 1 h in 50% methanol + 20% acetic acid, stained in Coomassie Brilliant Blue G dissolved in 10% acetic acid for visualization of the molecular weight marker, and destained in 10% acetic acid. Finally, the gel was dried and autoradiographed. The molecular weight marker was purchased from Sigma (catalogue number MW-SDS-17S). The molecular weight of Hok was estimated from a calibration curve of relative mobilities plotted against the known molecular weights of the seven marker polypeptides on semi-logarithmic paper.

Secondary structure probing

Modifications of *in vitro*-synthesized and gel-purified T7 Hok mRNA transcripts were performed as follows: DMS modification (modifies unpaired A and C residues with preference for A) was performed essentially as described by Moazed *et al.* (1986). 4 pmol RNA was incubated at 37°C in 10 μl DMS incubation buffer (80 mM sodium cacodylate pH 7.2, 20 mM $MgCl_2$, 100 mM NH_4Cl, 0.5 mM EDTA) containing 4 U RNasin. 1 μl 40 × diluted DMS (in ethanol) was added (in the control 1 μl ethanol was added) and the reaction was stopped after 5 or 20 min by addition of 20 μl DMS stop buffer (2 M ammonium acetate, 20 mM EDTA, 1 M β-mercaptoethanol, 2 μg/ml *E.coli* tRNA). It was then precipitated, washed and dried, resuspended in 200 μl 0.3 M NaOAc and extracted with phenol and $CHCl_3$.

CMCT modification (modifies unpaired U and G residues with preference for U) was performed according to Moine *et al.* (1988). 4 pmol RNA and 1.2 μg carrier tRNA were incubated at 25°C in 200 μl CMCT incubation buffer (50 mM sodium borate pH 8.0, 10 mM $MgCl_2$) containing 8 U RNasin. 50 μl CMCT (42 mg/ml dissolved in incubation buffer) was added and the sample was incubated for 3 or 20 min. In the control, 50 μl incubation buffer was added to the sample. It was then precipitated, washed, dried, resuspended in 200 μl 0.3 M NaOAc and extracted with phenol and $CHCl_3$.

Partial digestion of RNA with RNase V_1 (Pharmacia; specific for double-stranded helical regions or stacked nucleotides), RNase T_1 (Pharmacia; cleaves 3′ of G residues not involved in base-pairing) or RNase T_2 (Sigma; cleaves in single-stranded regions; no sequence preference) was performed basically as described in Öhman and Wagner, 1989. 4 pmol RNA in TMN binding buffer containing 10 μg/μl *E.coli* tRNA in a reaction volume of 5 μl was digested with 0.08 U V_1, 0.10 U T_1, or 2×10^{-3} U T_2 for 3 or 15 min at 37°C. In the control the RNA was incubated without enzyme. The reactions were stopped by immediate extraction with phenol (twice) and $CHCl_3$.

After modification, the RNA was precipitated, washed and resuspended in H_2O at a concentration of 0.2 pmol/μl.

The primer used for primer extension was HOK1 (5′-CGTGTC-AGATAAGTG-3′) complementary to nucleotides +369 to +383 in the Hok mRNA.

0.1 pmol [γ-^{32}P]ATP (NEN) end-labelled primer was annealed to 0.4 pmol RNA in 4.5 μl HEPES buffer (55.5 mM HEPES–KOH pH 7.0, 110 mM KCl) in the presence of 5 U RNasin (Promega) by heating to 50°C and slowly cooling to 40°C. Extension was performed at 42°C for 30 min after addition of 3 μl extension mixture [260 mM Tris–HCl pH 8.4, 20 mM $MgCl_2$, 20 mM DTT, 1.5 U AMV (avian myeloblastosis virus) reverse transcriptase (Life Science) and all four deoxynucleoside triphosphates at 110 μM]. For the sequencing reactions, 1 μl 40 μM ddATP/ddCTP or 1 μl 60 μM ddGTP/ddTTP was added additionally to the extension mixtures (performed on unmodified RNA). Reactions were stopped by ethanol precipitation and samples were resuspended in FD loading buffer (92% formamide, 17 mM Na_2EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue). Extension products were analysed on 6% polyacrylamide, 8 M urea sequencing gels.

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References

Blomberg,P., Wagner,E.G.H. and Nordström,K. (1990) *EMBO J.*, **9**, 2331–2340.

Carter-Muenchau,P. and Wolf,R.E. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 1138–1142.

Case,C.C., Simons,E.L. and Simons,R.W. (1990) *EMBO J.*, **9**, 1259–1266.

de Smit,M.H. and van Duin,J. (1990) *Progr. Nucleic Acids Res. Mol. Biol.*, **38**, 1–35.

Eguchi,Y., Itoh,T. and Tomizawa,J. (1991) *Annu. Rev. Biochem.*, **60**, 631–652.

Gerdes,K., Rasmussen,P.B. and Molin,S. (1986a) *Proc. Natl Acad. Sci. USA*, **83**, 3116–3120.

Gerdes,K. *et al.* (1986b) *EMBO J.*, **5**, 2023–2029.

Gerdes,K., Thisted,T. and Martinussen,J. (1990a) *Mol. Microbiol.*, **4**, 1807–1818.

Gerdes,K., Poulsen,L.K., Thisted,T., Nielsen,A.K., Martinussen,J. and Hove-Andreasen,P. (1990b) *New Biol.*, **2**, 946–956.

Gerdes,K., Nielsen,A., Thorsted,P. and Wagner,E.G.H. (1992) *J. Mol. Biol.*, **226**, 637–649.

Gold,L. (1988) *Annu. Rev. Biochem.*, **57**, 199–233.

Kameyama,L., Fernandez,L., Court,D.L. and Guarneros,G. (1991) *Mol. Microbiol.*, **4**, 1063–1067.

Kleckner,N. (1989) In Berg,D.E. and Howe,M.M. (eds), *Mobile DNA*. American Society for Microbiology, Washington, DC, pp. 251–263.

Miller,J. (1972) *Experiment in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Moazed,D., Stern,S. and Noller,H.F. (1986) *J. Mol. Biol.*, **187**, 399–416.

Moine,H., Romby,P., Springer,M., Grunberg-Manago,M., Ebel,J.P., Ehresmann,C. and Ehresmann,B. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 7892–7896.

Nielsen,A.K., Thorsted,P., Thisted,T., Wagner,E.G.H. and Gerdes,K. (1991) *Mol. Microbiol.*, **5**, 1961–1973.

Öhman,M. and Wagner,E.G.H. (1989) *Nucleic Acids Res.*, **17**, 2557–2578.

Persson,C., Wagner,E.G.H. and Nordström,K. (1988) *EMBO J.*, **7**, 3279–3288.

Petersen,C. (1989) *J. Mol. Biol.*, **206**, 323–332.

Rasmussen,P.B., Gerdes,K. and Molin,S. (1987) *Mol. Gen. Genet.*, **209**, 122–128.

Saito,H. and Richardson,C.C. (1981) *Cell*, **27**, 533–542.

Schägger,H. and von Jagow,G. (1987) *Anal. Biochem.*, **166**, 368–379.

Schleif,R. (1969) *Nature*, **223**, 1068–1069.

Sekiguchi,M. and Iida,S. (1967) *Proc. Natl Acad. Sci. USA*, **58**, 2315–2320.

Severinov,K., Soushko,M., Goldfarb,A. and Nikiforov,V. (1993) *J. Biol. Chem.*, **268**, 14820–14825.

Simons,R.W. and Kleckner,N. (1988) *Annu. Rev. Genet.*, **22**, 567–600.

Stark,M.J.R. (1987) *Gene*, **51**, 255–267.

Studier,F.W., Dunn,J.J. and Buzash-Pollert,E. (1979) In Russel,T.R., Brew,K., Farber,H. and Schultz,T. (eds), *From Gene to Protein: Information Transfer in Normal and Abnormal Cells*. Academic Press, Inc, New York.

Thisted,T. and Gerdes,K. (1992) *J. Mol. Biol.*, **223**, 41–54.

Thisted,T., Sørensen,N.S., Wagner,E.G.H. and Gerdes,K. (1994) *EMBO J.*, **13**.

Wikström,P.M., Lind,L.K., Berg,D.E. and Björk,G.R. (1992) *J. Mol. Biol.*, **224**, 949–966.

Zubay,G. (1973) *Annu. Rev. Genet.*, **7**, 267–287.

Zucker,M. and Stiegler,P. (1981) *Nucleic Acids Res.*, **9**, 133–148.

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