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The transcription of *NAM7/UPF1* is enhanced in the absence of Cyp1p/Hap1p concomitant with the appearance of an *ISF1-NAM7* cotranscript in *Saccharomyces cerevisiae*

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Abstract The two adjacent nuclear genes *ISF1* and *NAM7* cooperatively participate in mitochondrial functions. It is well known that Cyp1p(Hap1p) activates a number of genes involved in these same functions. We show in this paper that Cyp1p influences the transcriptional regulation of *NAM7*. In addition, a significant amount of *ISF1–NAM7* cotranscript is observed in a *cyp1* mutant context. An extensive analysis of the intergenic region which separates the two genes revealed 5′ starts of the *NAM7* transcripts, additional to those previously mapped. These new 5′ starts overlap the 3′ ends of *ISF1*. We propose that *NAM7* is under the control of a negative Cyp1p-dependent regulator and that its absence favours a transcriptional read-through which results in the *ISF1–NAM7* cotranscript we have identified.

Key words: Mitochondrial function; Nuclear gene; *CYP1(HAP1)*; Cotranscription; Transcriptional regulation

1. Introduction

The biogenesis of mitochondria depends on a set of complex regulatory mechanisms, which allow the products of both hundreds of nuclear and a few mitochondrial genes to interact within the organelle [1]. In yeast, a number of nuclear genes involved in these processes have been cloned by complementation of respiratory deficient mutants [2]. Alternatively such genes were identified by screening for multicopy suppressors of respiratory deficient mutants [3]. The latter approach allowed us to isolate a genomic fragment which, when amplified, is able to suppress mitochondrial intronic mutations located either in group I or group II introns [4]. This fragment was found to contain two adjacent genes, namely *ISF1* and *NAM7*. The latter, which encodes an RNA helicase with a Zn-ligand motif, is important for respiration and has been shown to be responsible for the suppressor activity [5]. The *NAM7* gene has been independently isolated as the *UPF1* gene which accelerates decay of mRNAs containing non-sense mutations [6] and the majority of its product has been found to be associated with polyribosomes [7,8]. The *ISF1* gene, which does not carry any suppressor activity by itself, has been found to behave as a helper of *NAM7* [9]. Hence, the full suppressor activity associated with the genomic fragment is the result of a cooperative effect of the two genes. Interestingly, the *ISF1* gene has also been isolated as *MBR3* in an independent study and found to be able to suppress the growth defect of *hap2*, *hap3* and *hap4* null mutants [10].

Several variables including glucose fermentation, oxygen tension in the growth medium and/or the availability to heme (it is generally admitted that heme transduces the oxygen signal [11]), are of particular interest because they all elicit significant changes in the levels of mitochondrial proteins of nuclear origin needed for the maintenance of a respiratory functional organelle. It has previously been shown that even though the *ISF1* and *NAM7* genes are involved in the same process, the *ISF1* gene, but not *NAM7*, is sensitive to fermentative repression, indicating that the two genes are not coordinately expressed.

A number of genes encoding trans-acting factors are involved in the O_2/heme-dependent regulation (for review see [12]). Among these, *CYP1* (also known as *HAP1* [13]) occupies a central position. Its product, Cyp1p, activates in O_2/heme sufficient conditions a number of genes involved in electron transfer mechanisms and respiration [14]. It also activates *ROX1* which encodes a heme-dependent repressor of hypoxic genes [15]. Thus Cyp1p behaves indirectly as a repressor of the genes regulated by Rox1p. We were therefore interested to investigate a possible involvement of Cyp1p in the expression of *ISF1* and/or *NAM7*.

2. Materials and methods

2.1. Yeast strains and transformation procedure

R23 has been described in [4]; wild-type VP209-7B (MATα, *ura3, leu2, cyc1-1)* and isogenic *cyp1* mutants VP209-7B/*CYP1-18* and VP209-7B/*cyp1::URA3* were described in [16]; wild-type AH12-7 (MATα, *trp1-1, cyc1-1, his4-519, leu2,-3, leu2-112, gal*) and isogenic AH12-7/*rox1::LEU2* are generous gifts of R. Zitomer. VNA is VP209-7B/*CYP1-18* carrying the *nam7::URA3* deletion in which an internal 1.7 kb *BglII* fragment of *NAM7* has been replaced by the *URA3* gene (this study). Deletion of the *NAM7* gene from the yeast chromosome was confirmed by Southern analysis of *SalI*/*Bam*HI-digested genomic DNA (not shown). Growth conditions and transformation procedure were the same as previously described [5,9].

2.2. E. coli transformation and plasmids

The plasmid pGRA31 is a derivative of pBR328 into which a 5.7 kb *SalI*/*Bam*HI genomic fragment carrying the *ISF1* and *NAM7* genes was cloned [5]; the M13 derivative MB probe has been described in [5]. The plasmids pNA and pNA8 are derivative of pGRA31 and carry respectively a *SalI*/*Msc*I or *SalI*/*Mun*I insert. pGSH1 is a pGEM11Zf plasmid (Promega) into which a *SalI*/*Hin*dIII fragment from pGRA31 was cloned [9]. Plasmids were propagated by transforming *E. coli* strains TG1 or JM101 made competent by $CaCl_2$ procedure [20].

2.3. Primer extension

The synthetic oligonucleotide 5′-TTAGTACGAAAGCGTATAA-AGGTGTCCCTCCC-3′ (see Fig. 3C) was labeled with [γ-^{32}P]ATP

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(>3000 Ci/mmol; Amersham) by T4 polynucleotide kinase (Boehringer-Mannheim) to a specific activity of 10^6 Cerenkov cpm/pmol. Labeled primer (10^5 cpm) was annealed with poly(A$^+$) RNAs, resuspended in 20 μl of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 4 mM Na-pyrophosphate, 0.25 mM each dATP, dCTP, dTTP, dGTP, 12 U of reverse transcriptase and incubated at 42°C for 60 min [20]. Extended cDNA fragments were analyzed by fractionation on a 6% acrylamide/7 M urea sequencing gel.

3. RNA preparation and analysis

Cells were grown on complete medium, 2% yeast extract, 1% bacto-peptone (Difco Laboratories) and 2% galactose, and arrested at OD=1. Total and poly(A$^+$) RNAs extraction, gel electrophoresis, Northern blotting and hybridization conditions were the same as described in [5]. RNase mapping was performed as described in [9]. Briefly, labeled riboprobe d was generated by digesting the plasmid pGSH1 with *Bst*XI and synthesizing RNA with SP6 polymerase in the presence of [α-^{32}P]CTP according to the instructions from supplier (Promega). Riboprobe was hybridized with 5 μg of poly(A$^+$) RNA at 45°C, 8–12 h, in 30 μl of 40 mM PIPES (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl and 80% formamide. Digestion of hybrids with RNase A and RNase T1 and following steps were performed essentially as described in [20].

4. Results and discussion

In order to look for a possible Cyp1p-dependent regulation of the transcription of the *ISF1* and *NAM7* genes, we purified poly(A$^+$) RNAs from strains VP209-7B and its isogenic derivatives carrying *cyp1::URA3* [16] or *CYP1-18*, a mutated allele described in [17]. We also used as a control RNAs purified from strain R23 from which the genomic fragment carrying *ISF1* and *NAM7* was originally isolated [4]. Northern blot experiments were performed and the *ISF1* and *NAM7* transcripts were revealed by hybridizing with a probe carrying both genes. This experiment led to the following conclusions. (1) The relative amount of the *ISF1* and *NAM7* transcripts is equivalent when RNAs are purified from the wild-type strains R23 and VP209-7B (Fig. 1, lanes 1 and 2). (2) The *NAM7* steady-state transcripts are increased by a factor of 3–4 in strains which carry either the *CYP1-18* allele or a deletion of *CYP1*, whilst those of *ISF1* are not affected (Fig. 1, lanes 3 and 4). Therefore, the *CYP1-18* allele and the deleted gene are equivalent concerning the regulation of *NAM7*. (3) Surprisingly, a new transcript of 5 kb, not detectable in the wild-type strains, is clearly present in the *cyp1* mutant strains.

These observations suggest that Cyp1p regulates negatively the *NAM7* and the 5 kb transcripts. Since it is known that Cyp1p activates the heme-dependent repressor *ROX1*, the negative regulation uncovered might be indirect and mediated by Rox1p. The Northern blot experiments were therefore repeated with RNAs purified from two isogenic *ROX1* and *rox1::URA3* strains. The results obtained (Fig. 1, panel B) show that neither the 5 kb transcript nor significant differences in the amount of the *NAM7* transcript are detected when RNAs were purified from a *rox1* null strain. We therefore concluded that the Cyp1p-dependent regulation we observed is not mediated by Rox1p.

The 5 kb transcript could be ascribed to the induction of a gene related but independent from *NAM7* or alternatively to a precursor either of *ISF1* or *NAM7* or both transcripts. In order to discriminate between these possibilities, we have deleted the *NAM7* gene in a *cyp1* mutant context and determined if the 5 kb transcript is affected as a consequence of the *NAM7* disruption. The disruption was performed as described in [5] and verified by Southern analysis of the genomic DNA (not shown). Poly(A$^+$) RNAs were purified from the disrupted strain and analysed by Northern blot. It can be seen in Fig. 1, panel A, that in this context, both the *NAM7* and the 5 kb transcripts are absent (lane 5), showing that the *NAM7* sequence is part of both transcripts. This was confirmed by the observation that both *ISF1* and *NAM7* probes are able to hybridize with the 5 kb transcript (see Fig. 2, lanes a and b). Moreover, a short probe, which corresponds to the

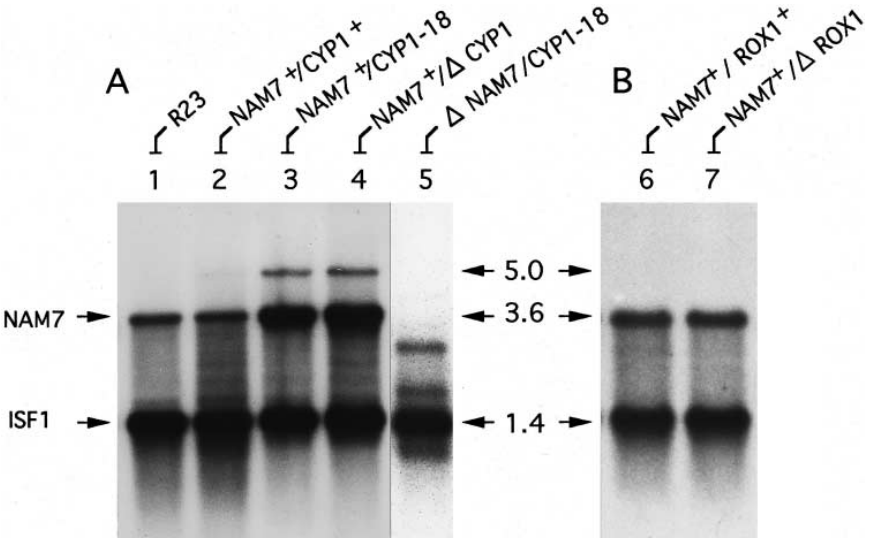

Fig. 1. Northern blot analysis of *ISF1* and *NAM7* transcripts in different mutants. 5 μg RNA of each preparation were loaded on denaturing agarose gel, blotted and revealed by hybridization with ^{32}P-labelled pGRA31 carrying both the *ISF1* and *NAM7* genes [9]. An actin probe was used as a standard (not shown). A: Lane 1, R23 strain; lane 2, wild-type (VP209-7B); lane 3, *CYP1-18* mutant (VP209-7B/*CYP1-18*); lane 4, *cyp1* deleted strain (VP209-7B/*cyp1::URA3*); lane 5, strain VNA (see Section 2). B: Lane 6, wild-type *ROX1*$^+$ (AH12-7); lane 7, *rox1* deleted strain (AH12-7/*rox1:LEU2*).

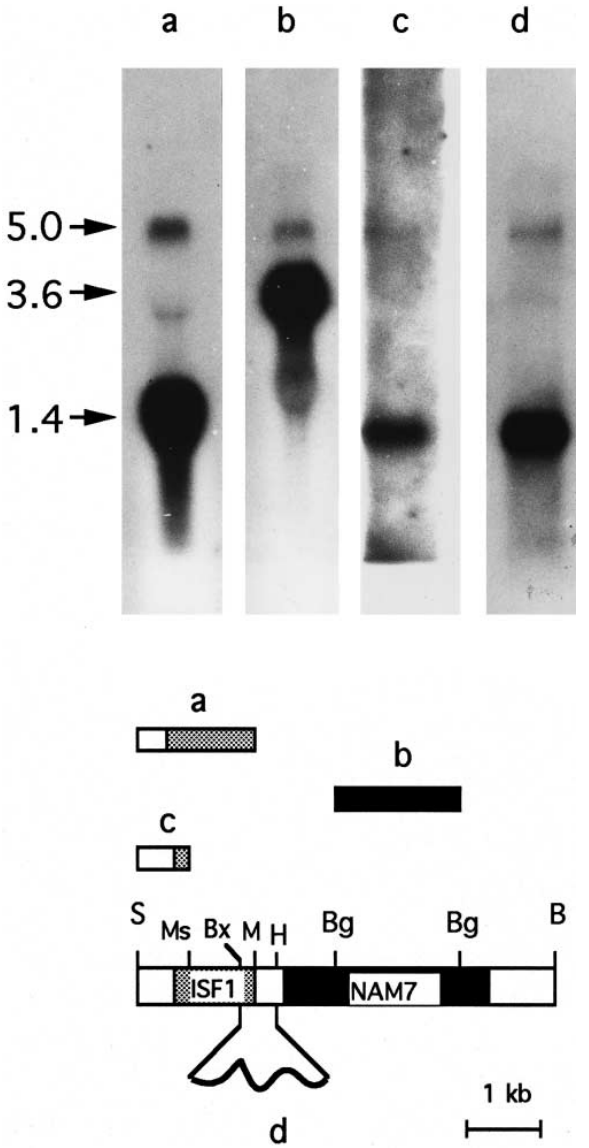

Fig. 2. Cross-hybridization of *ISF1* and *NAM7* specific probes with the 5 kb transcript. Poly(A$^+$) RNAs were extracted from the strain VP209-7B/*CYP1-18*. Blotted filters were separately hybridized with the corresponding probes depicted at the bottom. The restriction map of the 5.7 kb *Sal*I/*Bam*HI genomic fragment carrying both *ISF1* and *NAM7* is shown as well as the regions used as probes. Probes a (pNA), b (MB) and c (pNA8) are double-strand DNA. Probe d is an antisense RNA generated by run-off in vitro transcription of pGSH1 as described in Section 2. B = *Bam*HI, Bg = *Bgl*II, Bx = *Bst*XI, H = *Hin*dIII, M = *Mun*I, Ms = *Msc*I, S = *Sal*I.

5′ region of the *ISF1* coding sequence also hybridizes with the 5 kb transcript (Fig. 2, probe c, lane c). This observation strongly supports the idea that the 5 kb transcript includes both *ISF1* and *NAM7*. However, since we used double-strand probes, we could not exclude the possibility that the long transcript is revealed by hybridization with the non-coding strand. We therefore generated an antisense RNA which includes the 3′ part of the coding sequence of *ISF1* and a large part of the *ISF1-NAM7* intergenic region. As shown in Fig. 2, lane d, this riboprobe hybridizes with both the *ISF1* and 5 kb transcripts. In addition to these, a weak but significant signal, corresponding to the *NAM7* transcript is also detected.

In a previous study we mapped the 3′ termini of *ISF1* and the 5′ starts of *NAM7* within the intergenic region and showed that they are independent. In particular, several 5′ starts of *NAM7* determined, either by RNase protection or primer extension, were localized within a range of 70–90 nucleotides upstream the *NAM7* ATG codon [9]. In this study, we show that a riboprobe covering a region located upstream of the *Hin*dIII site (position 1941, Fig. 3, panel C) is hybridizing with the *NAM7* transcript, indicating the presence of 5′ ends additional to those detected in the previous experiments. In order to get a better insight into the intergenic organization, we synthesized an oligonucleotide upstream the *Hin*dIII site to be extended as cDNA on the *NAM7* transcript template. In the primer extension experiment shown in Fig. 3, panel B, several stops of the reverse transcriptase are detected. The 5′ terminal nucleotides of the most abundant cDNAs are located essentially in the same region as the most abundant 3′ ends of *ISF1* which have previously been mapped. RNase protection experiments (Fig. 3, panel A) show that the 3′ termini of *ISF1* are identical in a *CYP1*$^+$ and a *cyp1* mutant context. It therefore appears that the 3′ termini of *ISF1* and the newly mapped 5′ starts of the *NAM7* transcript overlap. In addition, as expected in this last experiment, a larger RNase protected fragment corresponding to the 5 kb transcript is detectable only in a *cyp1* mutant context.

The original goal of our work was to look for a Cyp1p-dependent regulation of the *ISF1* and *NAM7* genes. We show here that the transcription of *NAM7* is indeed enhanced in the absence of Cyp1p, concomitant with the appearence of an *ISF1–NAM7* cotranscript. A model according to which the *ISF1* and *NAM7* transcripts merely result from the processing of an intermediate cotranscript requires additional functions for Cyp1p in both mRNA stability and RNA processing. In a previous study, we have shown that in contrast to *ISF1*, *NAM7* is insensitive to catabolite repression [9]. This favours the idea that *NAM7* is regulated independently from *ISF1*. The fact that the *NAM7* transcripts are specifically enhanced in *cyp1* mutant strains suggests that a negative regulatory factor able to bind the intergenic region could be missing. A careful mapping of the *ISF1–NAM7* intergenic region led to the observation that at least part of the 5′ ends of the *NAM7* transcripts overlap the extremities of *ISF1* (Fig. 3, panel C). This unusual organization of the intergenic region might facilitate the occurrence of a transcriptional read-through, especially if the region is partially or totally devoid of regulatory factors, in the absence of Cyp1p. It is of interest to note that the longer *NAM7* transcripts mapped here include a short open reading frame (ORF) which overlaps the first 10 codons of the *NAM7* ORF (Fig. 3, panel C). Intriguingly, transcripts with small upstream ORFs have been suggested to undergo a destabilization mechanism mediated by Nam7p(Upf1p) itself [8].

Although nuclear polycistronic transcripts are rare in eucaryotes, they are well known in viruses of eucaryotic cells and have recently been found in organisms such as trypanosomes and *Caernorhabditis elegans* (see [18] and references therein). In yeast a detailed analysis of the transcript region of *PET122* also revealed overlapping transcription [19]. It is therefore possible that a more elaborate analysis of other transcripts would reveal similar pattern of transcription. Whatever the case, the study of the transcriptional regulation of *NAM7* should deepen our understanding of the mechanism involved

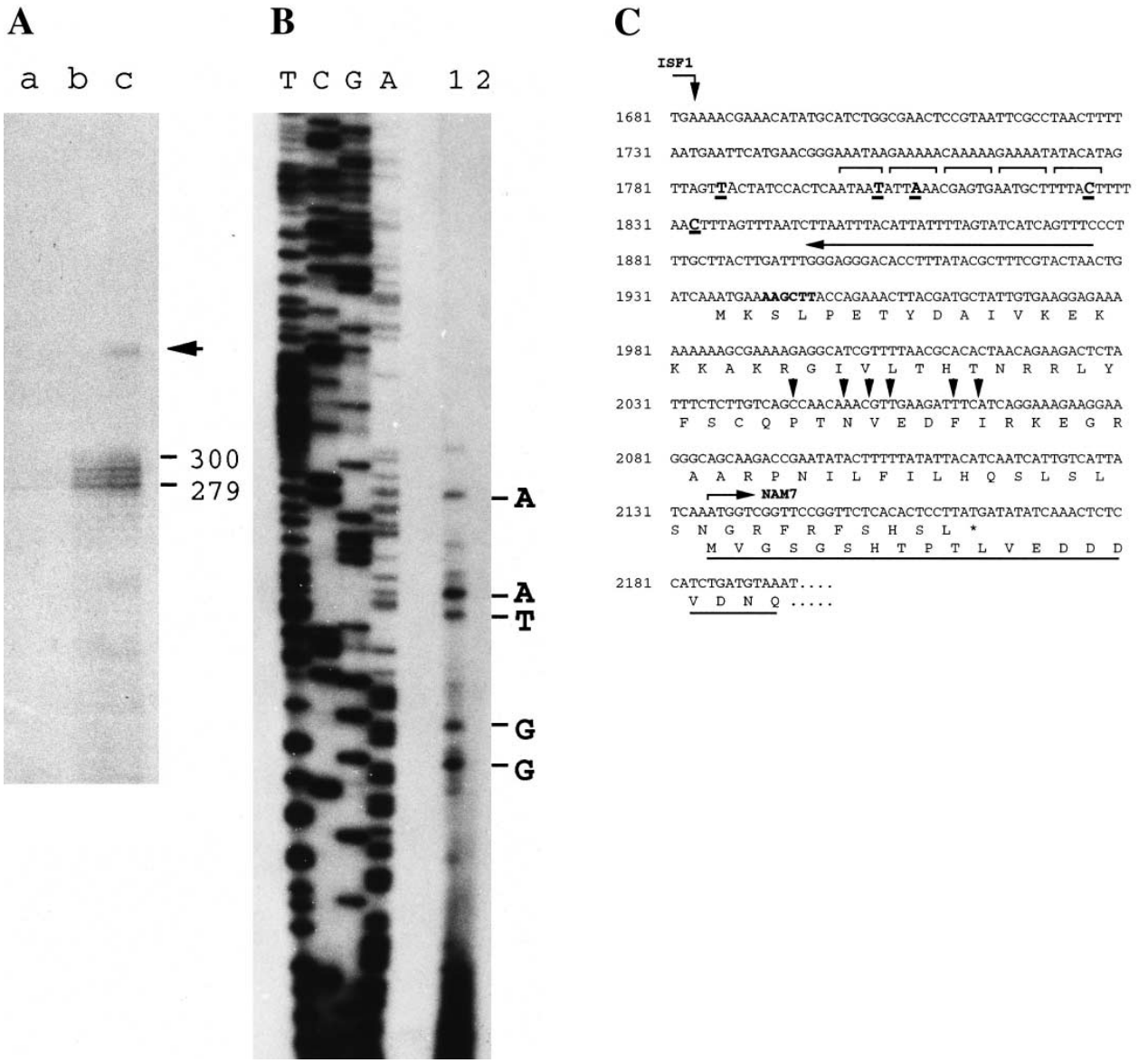

Fig. 3. Mapping of the *ISF1* 3′ and *NAM7* 5′ termini. A: The *ISF1* 3′ ends were mapped in both the wild-type and VP209-7B/*cyp1::URA3* strains for comparison. Poly(A$^+$) RNAs was hybridized with the riboprobe d depicted in Fig. 2. The most abundant protected fragments are shown. The maximum and minimum size of the fragments is indicated in nucleotides. Arrow indicates the additional larger protected fragment in the *cyp1* deleted strain. Lane a = tRNA control; lane b = wild-type; lane c = VP209-7B/*cyp1::URA3*. B: Primer extension of the *NAM7* transcript. The synthetic oligonucleotide indicated by the arrow overline in (C), was used to synthesize cDNA on poly(A$^+$) RNAs. Extended cDNA fragments were analyzed by fractionation on a sequencing gel alongside dideoxy sequence products elongated using the same labeled primer on pGSH1 DNA template. Initial nucleotides of the most abundant cDNA termini are indicated. Lanes T-A = sequencing reactions; lane 1 = extension products; lane 2 = heterologous tRNA control. C: Localization of 3′ ends of *ISF1* and 5′ starts of *NAM7* within the intergenic region. The TGA stop codon of the *ISF1* and the ATG start of the *NAM7* ORFs are shown. The 3′ ends of *ISF1* are indicated by bars above the sequence. The 5′ starts of the *NAM7* transcripts, as determined here by primer extension and those previously mapped [9], are indicated in underlined bold letters and arrows respectively. The *Hin*dIII site is indicated in bold letters. The *NAM7* protein sequence in one letter code is underlined. A short ORF is also shown upstream of this latter.

in the cotranscription of the two adjacent genes, *ISF1* and *NAM7*, which have previously been shown to participate cooperatively to the same process [9].

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