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Transcriptional terminators in the *caa-cal* operon and *cai* gene

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ABSTRACT

We have analysed by S1 nuclease mapping the *in vivo* termination sites of transcription of the *caa-cal* operon and *cai* gene. The termination region for *caa* mRNA (T1A terminator) features characteristics of a rho-independent terminator. This terminator is a convergent transcription terminator, its complementary secondary structure being present at the 3'-end of *cai* mRNA. The *caa-cal* mRNA terminator (T2A terminator) has a stable potential secondary structure and shows homology with rho-dependent terminators. *In vitro* transcription of *caa-cal* operon demonstrated that the two terminators TIA and T2A are efficient. The 3'-ends of the mRNAs which end at T1A and T2A were analysed by S1 mapping with total RNA purified from a mutant strain deficient in exoribonuclease activities, in particular RNase II. The results suggest that the potential secondary structures of T1A and T2A are sufficiently stable to prevent 3'-end degradation by RNase II. On the other hand, the T2A terminator should be efficient enough to stop transcription through the downstream DNA region involved in pColA replication.

INTRODUCTION

Three proteins encoded by the ColA plasmid have been characterized with respect to their functional properties: colicin A (encoded by the *caa* gene) is a pore forming protein (1), the immunity protein (*cai* gene) which protects producing cells from colicin A (2) and the lysis protein (*cal* gene) whose action is required for the extracellular release of colicin A (3). These genes span a region of about 2700 nucleotides out of the 6720 bp of the ColA plasmid (4). We have previously demonstrated that *caa* and *cal* are organized in an operon (5). This operon is under the control of the LexA repressor and thus transcription can be induced with DNA damaging agents like mitomycin C (MTC). Two overlapped LexA boxes were demonstrated by DNase I footprinting experiments and located just downstream of the transcription initiation site of the *caa-cal* operon (5). The *cai* gene is located in the intercistronic region and transcribed in the opposite direction (5).

The promoter of the *caa-cal* operon is very efficient and for example, after induction, colicin A can account for more than 30% of total cell protein synthesized (6). The Cal protein can also cause early cell death when it is overproduced, thus the T1A terminator located between *caa* and *cal* plays an important role. In addition the possibility existed that this terminator may function bidirectionally since the transcription of *caa* and *cai* is convergent. Just downstream

from this operon, the region encoding the RNA II (or RNA primer for plasmid replication) is located (4). The T2A terminator of the *caa-cal* mRNA thus separates two DNA regions of pColA. For all of these reasons, it was of interest to analyse the termination sites and to obtain some information on the exact location and the nature of the terminators.

MATERIALS AND METHODS

Strains and plasmids

We used *E.coli* K12 derivative strains: C600, CAN/20-12E (lacking RNases BN, II, D, I) and the wild type parent strain CA265 (7). These last two strains were kindly provided by Dr. Deutsher. Plasmids pColA, pColA9 (which differs from pColA by the addition of the *bla* gene and the origin of replication of pBR322), and pDV5 (which differs from pColA9 by a modification of the *caa-cal* promoter which results in a very low level of transcription) have been previously described (5). pBC7 was obtain from pColA9 after a deletion of 2/3 of the 3'-end of the *caa* coding sequence as described (6).

RNA purification

Total RNA was purified as described (5) except for the growth conditions and induction. C600 (pColA) was grown in M9 minimal medium and induced at OD_{600nm}= 0.1 with 100ng/ml of MTC. C600 (pColA9), CA265 (pColA9) and CAN/20-12E (pColA9) were grown in LB medium with addition of MTC (300ng/ml) at OD_{600nm}= 0.5.

S1 mapping of termination sites and labelled probes

Purified total *E.coli* RNAs were analysed by S1 nuclease mapping using 3'-end labelled probes. The probes were labelled with the filling activity of the Klenow fragment of the DNA polymerase I (3 units) in 10mM Tris-HCl pH 7.5, 10mM MgCl2, 1mM DTT 30 min at 20°C with (α^{32}P)dATP (3000 Ci/mmol, 10 mCi/ml) and the three other dNTPs. For DNA labelling at the *HincII* site (position 2603, see **fig.1**), we used the two activities of the Klenow fragment and blocked the exonuclease activity by adding only dCTP and (α^{32}P)dATP nucleotides. (In this case no A or C nucleotides were present at the cleavage site, the first A nucleotide being located 5 bases from the cleavage site and preceded by a C nucleotide.) The probe was then hydrolysed to obtain only one 3'-end labelled extremity. S1 mapping analysis was performed as previously described (5) except that we used double stranded probes and the hybridization mixture was incubated in 80% deionized formamide instead of 50%.

In vitro transcription

3 nM of template DNA (plasmid BC7 linearized at *PvuII* or *PvuII* and *NcoI*) was incubated for 10 min at 37°C in 20 mM Tris-acetate, pH 7.9, 150 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 10 mM MgCl2 with or without the LexA protein (500 nM). RNA polymerase holoenzyme (2 units), 150 µM ATP, GTP and CTP, 15 µM of UTP and 1 µl of (α^{32}P) UTP(410 Ci/mmol, 1 mCi/ ml) were added and the mixture was incubated 10 min at 37°C. Then 150 µg/ml heparin was added and the mixture incubated a further 10 min. The mixture was

phenol extracted, ethanol precipitated twice and analysed by electrophoresis on a 8.3 M urea, 5% polyacrylamide gel.

Northern blot analysis

Denaturing formaldehyde agarose gels (1%), buffer and samples were prepared according to Maniatis *et al.* (8). After electrophoresis, the gel was transfered by electrophoretic blot onto a nylon filter (Amersham, Hybond N) in 25 mM Sodium Phosphate, pH 6.5. Total RNA was fixed by U.V. crosslinking. The filter was prehybridized and probed with oligonucleotide as described (9). Two 15 mer oligonucleotides were synthesized using phosphoramidite chemistry on an Applied Biosystems DNA synthesizer, model 381A. These oligonucleotides contained sequences from the *caa* coding region (position 1533 to 1547 for the *caa*-oligonucleotide) and from the *cal* coding region (position 2669 to 2683 for the *cal*-oligonucleotide).

Enzymes

LexA protein was kindly provided by Dr. Schnarr (IBMC, Strasbourg). RNA polymerase holoenzyme was obtained from New England Biolabs, Inc. and S1 nuclease from Gibco Bethesda Research Laboratories. Other enzymes were purchased from Boehringer Mannheim.

RESULTS

Transcription termination of the *caa-cal* operon

The 3'-end labelled double stranded probes, used in S1 mapping to locate the termination sites of transcription were *NdeI*(1816)-*HincII*(2124) for *caa*, *NdeI*(1816)-*EcoRV*(2896) for the *caa-cal* operon and *HincII*(2603)-*EcoRI*(2896) or *BglII*(2190)-*PstI*(3837) for *cal* (**fig.1**). In order to quantify the termination signal, in some experiments, we used the 5'-end labelled *NcoI*(259)-*PvuII*(5238) probe to evaluate the ratio between the initiation of *caa-cal* operon and transcription termination. Total RNA from C600 cells bearing pColA was purified

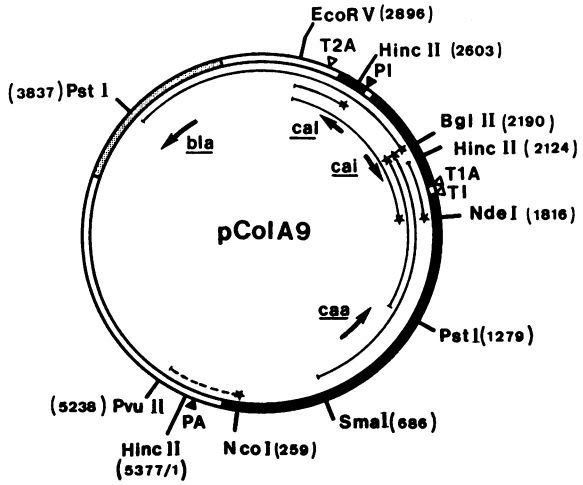

Fig.1: The 3'-end labelled probes used in S1 mapping to locate the transcription terminator sites. The stars represent the 3'-end labelling. The dashed line indicates a 5'-end labelled probe. T1A, T2A and TI are respectively the terminator sites for the *caa* gene, *caa-cal* operon and *cai* gene. PA and PI are respectively the Pribnow boxes of the *caa-cal* operon and *cai* gene. The restriction enzyme sites used for the purification of the probes are indicated.

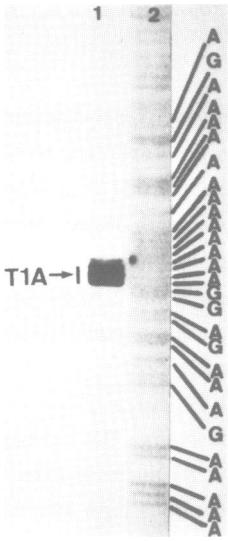

Fig.2: S1 mapping of the transcription terminator of *caa*. The *NdeI-HincII* 3'-end labelled probe was hybridized with total RNA purified from C600 (pColA) cells treated 5 hours with MTC. Lane 1 shows the specific signals of T1A, and lane 2 the A plus G nucleotide sequence of the probe.

after addition of MTC to the culture medium to induce the transcription of the *caa-cal* operon. The transcriptional termination region of *caa* mRNA was determined by S1 mapping with the *NdeI-HincII* probe and localized using the nucleotide sequence of this probe (after a correction of 1 base for the Maxam and Gilbert sequencing reaction (5)). Three major S1 nuclease resistant hybrids were reproducibly obtained (**fig.2**, lane 1). These termination signals were located at the beginning of the poly U region of the *caa* mRNA, 27 bases downstream from the coding sequence of the *caa* gene (see T1A signals, **fig.3**). We used the *NdeI-EcoRV* probe to check if there were any S1 resistant hybrids located after T1A. Nothing was detected in the intercistronic region between *caa* and *cal*. The T2A signal was only observed with RNA from MTC-treated cells as was observed for T1A signals (**fig.4A**, lanes 1, 2). We demonstrated that T2A was the transcriptional terminator of the *caa-cal* operon. To obtain a better resolution for T2A, the 3'-end labelled probe *HincII-EcoRV* was used. With this shorter probe the S1 mapping results indicated three distinct signals inside T2A when the operon was induced for 5 hours. These signals are called T2Aa, T2Ab and T2Ac (**fig.4B**). There was no other major S1 signal between the stop codon of *cal* and T2A (in which T2Aa is located 49 bases downstream from the stop codon). We observed that during induction the first major signal was the shorter one (T2Aa). After 5 hours the longer one (T2Ac) became the major one (**fig.4B**, compare lane 1 to lane 2). To check that there was no artifact of the S1 mapping technique, for example due to the run of 6A nucleotides inside T2A, total denatured RNA was hybridized at 20°C (instead of 42°C) with the *HincII-EcoRV* probe in order to stabilize the hybrids (10). No difference with the standard condition was observed (data not shown), in particular we did not notice any stabilization of the longer transcript (T2Ac) during hybridization at 20°C. Therefore, we do not

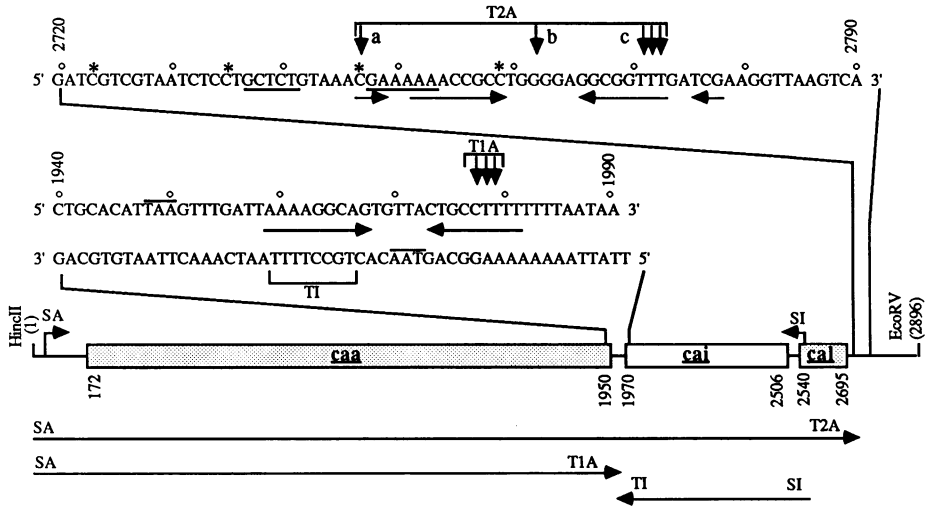

Fig.3: Localization of the transcription termination signals of the *caa-cal* operon and *cai* gene. The position of the ATG and stop codons are indicated for each gene. SA, T1A and T2A indicate respectively the transcriptional start site and the two termination sites of the *caa-cal* operon. The three major signals a, b and c inside T2A are indicated. SI and TI are the initiation and termination sites of *cai* transcription. The stop codons of *caa* and *cai* are overlined. The stable secondary structures of 3'-end mRNA are represented by inverted arrows. These inverted arrows correspond to the potential terminators T1A, T2A and TI. The sequences underlined correspond to specific sequences found in other rho-dependent terminators. Vertical arrows indicate the position of the major S1 resistant hybrids. The stars correspond to the C nucleotides found every 12 bases upstream of T2A (3 other C nucleotide separated by 11 bases are located upstream of the position 2720).

know if the three signals (a, b and c) inside T2A correspond to three distinct transcripts or if there is 3'-end degradation of the longer transcript T2Ac.

The two transcripts SA-T1A and SA-T2A of the *caa-cal* operon (**fig.3**) were detected by northern blot analysis using two 15 mer oligonucleotides as probes (**fig.4C**). The two mRNA transcripts (SA-T1A: 1944 bases and SA-T2A: about 2710 bases, see **fig.3**) were observed with the *caa*-oligonucleotide (**fig.4C**, lanes 3, 4) and only the longer transcript was detected with the *cal*-oligonucleotide (**fig.4C**, lanes 1, 2). However, SA-T2A mRNA migrated with the 23S ribosomal RNA and this resulted in problems during the filter hybridization and caused distorted bands. This probably explains the apparent very low level of SA-T2A mRNA as compared to SA-T1A mRNA (**fig.4C**, lanes 3, 4) in comparison to the termination signals obtained by S1 mapping (**fig.4A**, lanes 1 and 2).

Transcription termination of the *cai* gene: a convergent transcription terminator

We used either the *BglII*(2190)-*PstI*(1279) or *BglII*(2190)-*SmaI*(686) probes to locate the termination site of transcription of the *cai* gene (**fig.1**). We have previously shown that the effect of convergent transcription of *caa-cal* operon decreases the level of *cai* transcription (5).

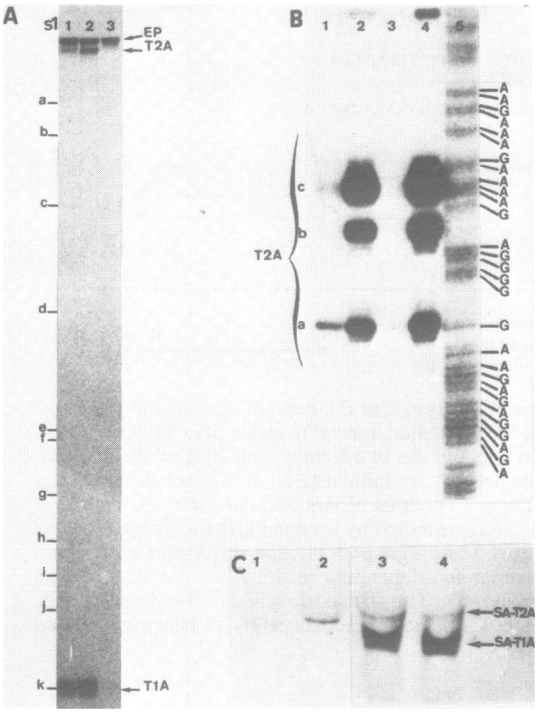

Fig.4: The *caa-cal* operon termination sites. **A.** The S1 resistant bands T1A and T2A were obtained after hybridization of the *NdeI-EcoRV* probe with total RNA from C600 (pColA) cells treated 5 hours with MTC (lane 1, 3 µg and lane 2, 6 µg of total RNA were used) or untreated (lane 3, 3 µg). The size markers pBR322 cut with *HpaII* are represented in lane s1 (corresponding sizes: a, 622; b, 527; c, 404; d, 309; e, 242; f, 234; g, 217; h, 201; i, 190; j, 180; k, 160; l, 147 bp...). T1A and T2A are respectively the terminators of *caa* and *caa-cal*. EP corresponds to the entire probe. **B.** S1 mapping of T2A with the 3'-end labelled *HincII-EcoRV* probe. The S1 resistant bands were obtained after hybridization with total RNA from C600 (pColA) treated 1.5 hour (lane 1), 5 hours (lane 2 and 4) or untreated (lane 3). 3 µg of total RNA were used except in lane 4 (6 µg). The sequencing reaction of the probe at nucleotides A and G is indicated (lane 5). a, b and c correspond to the 3 major signals of T2A. **C.** Northern blot of total RNA from C600 (pColA) cells treated with MTC for 3 hours (lanes 1 and 3) or 5 hours (lanes 2, 4). After hybridization, the first oligonucleotide, located inside the *cal* gene (lanes 1 and 2) was washed at 49°C and the second, in the *caa* gene (lanes 3, 4) was washed at 55°C. The lower arrow corresponds to the *caa* mRNA (SA-T1A) and the upper to *caa-cal* mRNA (SA-T2A).

To locate the *cai* termination signal we used C600 (pDV5) cells. This plasmid, in which the promoter of *caa-cal* was partially deleted, gave a very low basal level of transcription of the operon thus allowing increased constitutive transcription from the convergent *cai* gene. The S1

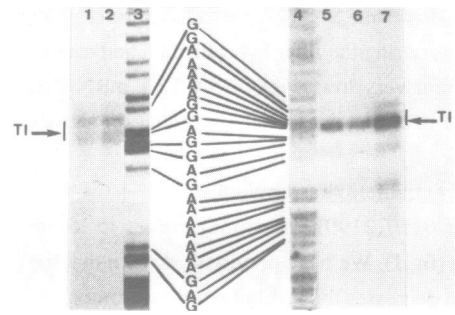

Fig.5: S1 mapping of TI. The 3'-end labelled *BglII-PstI* probe (*PstI* at position 1279 in fig.1) was hybridized with total RNA from C600 (pColA9) treated with MTC for 2 hours (lane 1) or non treated (lane 2). The G nucleotide sequence is shown in lane 3. The 3'-end labelled *BglII-SmaI* probe was used for S1 mapping with RNA from C600 (pDV5) treated 2 hours with MTC (lane 5) or untreated (lane 6). Twice as much was loaded in lane 7 as compared to lane 5 in order to insure an excess of probe. Lane 4 shows the sequence of the probe at A and G nucleotides.

mapping results obtained with the RNA from C600 (pDV5) (**fig.5**, lanes 5 to 7) indicated that the termination signals were located at the end of the potential stem and loop structure of the *cai* mRNA, which is complementary to the *caa* terminator. The same result was obtained with C600 (pColA) or C600 (pColA9) (**fig.5**, lanes 1, 2). According to the purity of the probe and its specific activity, the conditions of S1 mapping varied in the range of 20 to 50 µg of total RNA hybridized to detect the very low level signal of TI (about 20 times less was used for T1A or T2A). At present we have not yet investigated the relative efficiency of this terminator in the

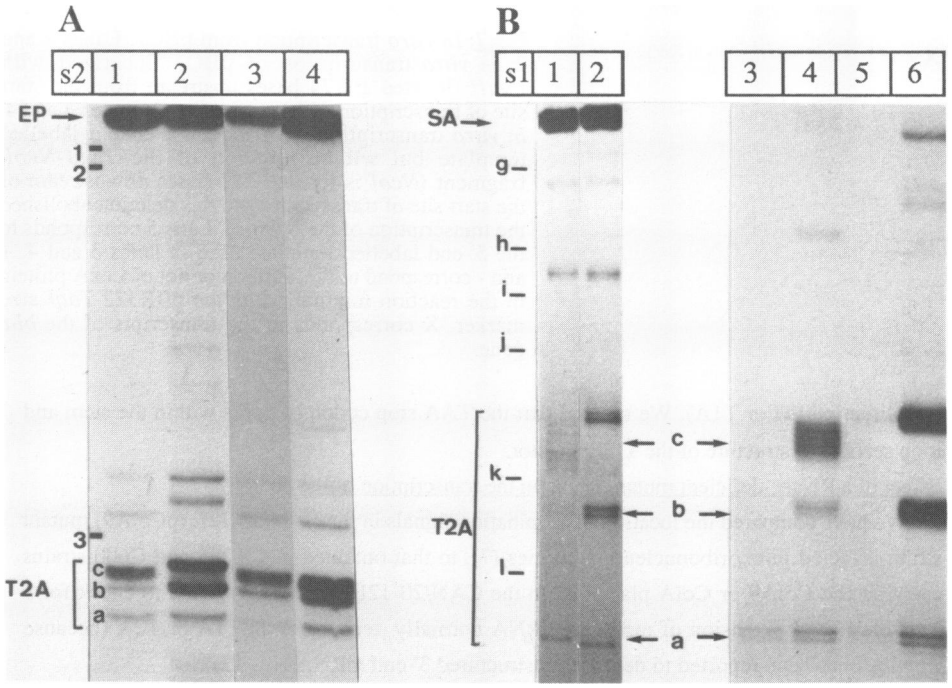

Fig.6: Comparison of T2A in pColA and pColA9. Effect of the defective RNase strain CAN/20-12E on termination. The size markers represented in lanes s1 and s2 correspond respectively to pBR322 hydrolyzed with *HpaII* and pBR322 hydrolyzed with *TaqI* restriction enzymes (lane s2, corresponding sizes: 1, 1444; 2, 1307; 3, 616 bp...). **A.** The 3'-end labelled probe *BglII-PstI* (*PstI* at position 3837 in fig.1) hybridized with the corresponding RNA was hydrolyzed 2 hours (at 25°C) with 300 units of S1 nuclease. EP corresponds to the entire probe. Total RNA was purified 3 hours after MTC treatment from CA265 (pColA9), lane 1; CAN/20-12E (pColA9), lane 2; C600 (pColA), lane 3 and C600 (pColA9), lane 4. **B.** Quantitative analysis of T2A with RNA from CA265 (pColA9) (lanes 1, 3 and 4) and CAN/20-12E (pColA9) (lanes 2, 5 and 6). For quantitative S1 mapping of T2A, the 3'-end labelled *HincII-EcoRV* probe was used with the 5'-end labelled *NcoI-PvuII* probe (lanes 1, 2) which mapped the start site of transcription of *caa-cal* operon (SA in fig.3). In lanes 3 and 5, the results were obtained with RNA purified after 1.5 hour of MTC treatment instead of 3 hours of treatment in the other lanes.

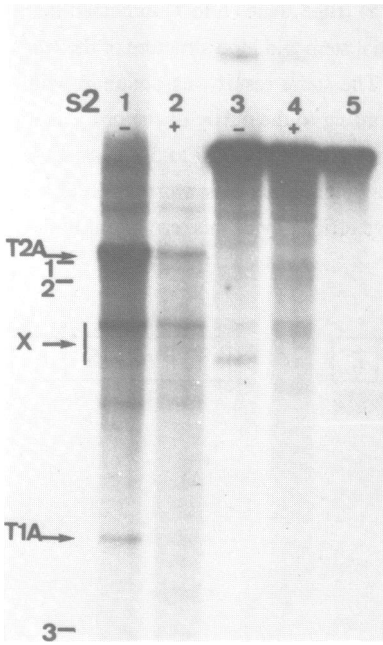

Fig.7: *In vitro* transcription from pBC7. Lanes 1 and 2: *in vitro* transcription of pBC7 linearized with *PvuII* (located at 174 bases upstream from the start site of transcription of *caa-cal* operon). Lanes 3 and 4: *in vitro* transcription with the same 5'-end labelled template but with a deletion of the *PvuI-NcoI* fragment (*NcoI* is located 223 bases downstream of the start site of transcription, so this deletion abolished the transcription of the operon). Lane 5 corresponds to the 5'-end labelled template used in lanes 3 and 4. + and - correspond to the addition or not of LexA protein in the reaction mixture. s2 is the pBR322 *TaqI* size marker. X corresponds to the transcripts of the *bla* gene.

two direction (TI or T1A). We noticed that the TAA stop codon of *cai* is within the stem and loop secondary structure of the TI terminator.

Effect of a RNase-deficient mutant strain on the transcription of the operon

We have compared the location of termination signals in the CAN/20-12E (pColA9) mutant strain affected in exoribonuclease activities (7), to that obtained in CA265 and C600 strains carrying the ColA9 or ColA plasmids. In the CAN/20-12E strain, we wanted to check for a possible 3'-end extension of messenger RNA normally terminated at T1A or T2A (because RNase II has been reported to degrade unstructured 3'-end mRNA (11, 12)).

The results obtained for T1A indicated no difference in the location of the S1 signal, moreover no significant variation was observed in the amount of transcripts for CAN/20-12E (pColA9) compared to the other strains cited above (data not shown). However, for T2A there was a difference when the RNase mutant strain was used (**fig.6A**, lane 2 and **fig.6B**, lanes 2, 5 and 6). This difference was detected only for the longer signal T2Ac, which was extended by a few nucleotides (**fig.6B**). The potential secondary structure of the T2A terminator (see the inverted arrows in **fig.3**) should certainly stabilize it against 3'-end degradation in the wild type strain (with RNase II activity). This situation of protection against 3'-end degradation by a stable secondary structure of mRNA has been observed for the *trp* operon (12). To check that there was no degradation of the SA-T2A mRNA up to T1A (see **fig.3**), we used quantitative S1 mapping in order to compare T2A termination in the mutant RNase and wild type strains.

We scanned the S1 resistant hybrids corresponding to the initiation site (SA) and termination sites (all the signals inside T2A) of *caa-cal* transcription and calculated the ratio T2A / SA. We did it this way because it is not possible to quantify in a reliable way the ratio of T1A termination to T2A termination by S1 mapping. Indeed, for S1 mapping of the 3'-end of the mRNA, the same probe (for example the *NdeI-EcoRV* probe) can hybridize with the shorter transcript SA-T1A and also with the T1A-T2A region of the mRNA (corresponding to the intercistronic and *cal* mRNA) which is contained in the longer transcript SA-T2A. S1 nuclease treatment would have decreased the longer signal and thus increased the ratio of the shorter transcript T1A compared to T2A. After scanning (**fig.6B,** lanes 1 and 2), similar ratios T2A / SA were obtained with RNA from CAN/20-12 E (pColA9) or CA265 (pColA9). This means that no 3'-end degradation occurred upstream of the signal T2A. After 3 hours of induction the major signal was in each case the longer one T2Ac, while after 1.5 hour of induction the major signal was always the shorter one (T2Aa). We have compared the T2A signals from pColA to that of pColA9 (**fig.6A**, lanes 3 and 4). In constructing pColA9, we have changed the context downstream from the *caa-cal* operon by deleting a part of the replication region of pColA. Thus there could be a difference for the T2A transcription terminator. In fact, the same location was observed for the T2A signals of the two plasmids.

In vitro transcription of *caa-cal* operon

In order to verify if the T1A and T2A termination signals observed *in vivo* were efficient *in vitro*, run off transcription was carried out with the plasmid pBC7 (cut at different sites) in the presence or absence of LexA protein. About 1270 bp of the *caa* coding sequence are deleted in pBC7 allowing higher resolution to be obtained in the analysis of the size of the transcripts. We have previously shown that the same transcriptional initiation site (SA) was obtained *in vivo* and *in vitro* (5), thus if the same terminators operate in this plasmid, this template should give two transcripts (from SA) of 674 and 1454 bases. These predicted transcripts were indeed obtained with pBC7 linearized at *PvuII*, and confirmed that the same terminators are functional *in vitro* as *in vivo* (**fig.7**, lane 1). T1A could be detected only in the absence of the LexA protein in the reaction mixture. For T2A, the signal was decreased in the presence of the LexA protein (**fig.7**, lane 1 compare to lane 2). Nothing was detected at the predicted sizes in the absence (or presence) of the LexA protein with the template pBC7 deleted for the *caa-cal* promoter (**fig.7**, lanes 3, 4). The bands called X could proceed from the *bla* transcripts (between 1000 and 1150 according to Gabain *et al* (13)). In pBC7 we changed the context upstream from T1A and the position of the stop codon, and this might have affected the efficiency of T1A as compared to the wild type operon.

DISCUSSION

For the *caa-cal* operon, two terminators of transcription were detected. The T1A terminator, located upstream of T1A signals (**fig.3**), has a potential stem and loop secondary structure

followed by a run of 8 U residues and thus corresponds to a rho-independent terminator (14,15). We have scanned the signals of T1A and T2A (**fig.4A** lanes 1 and 2). The result indicated that about half of the transcript initiated at SA were arrested at T1A. This terminator was perhaps less efficient than expected because the distance between the stop codon and the potential secondary structure of T1A is only 8 bases. Johnston and Roth (16) have shown that ribosomes can interfere with mRNA secondary structure 14 bases downstream. However, the *in vitro* results using pBC7 as template demonstrated that the T1A terminator functions without additional factors.

The very low level of transcription of the convergent *cai* gene located in the intercistronic region of *caa-cal* makes any estimation of the termination efficiency by S1 mapping difficult. The TI termination signals were detected after the same but complementary putative secondary structure as that contained in T1A. This TI terminator thus appears to be a convergent rho-independent transcription terminator. The run of only 4 U nucleotides at the 3'-end of TI may result in a less efficient termination than that observed for T1A. The short distance between the *cai* stop codon and the site of transcription termination could interfere with secondary structure formation. On the other hand, we noticed that the terminator of *cai* is located after a specific sequence for rho-dependent termination at position 2012-2007 (CACACC: "element IV" according to Morgan *et al.* (17)) in a 3'-end mRNA rich in A and T nucleotides.

By S1 mapping we observed three termination signals within the T2A terminator. The stability of the messenger RNA secondary structure (according to Zuker and Stiegler (18)) was calculated on a window of 200 bases covering each of the 3 possible transcripts terminated at T2A (T2Aa to T2Ac). Only the longer transcript T2Ac was found to be terminated at a potentially stable secondary structure (**fig.3**). With the RNase mutant strain CAN/20-12E (pColA9) T2Ac was extended by a few bases. These results indicated that in the wild type strain, RNase II may degrade a few bases and then is stopped by the strong hairpin structure of T2A. The T2A terminator involves a GC rich stem with a run of 3 U residues. This situation can be compared to that of the *tonB-P14* bidirectional rho-independent terminator described by Postle and Good (19). These authors have emphasized the idea of an extended symmetry that may enhance termination efficiency in the absence of a long run of U nucleotides. The T2A rho-independent terminator may function in both directions, in which case it would also stop RNA I convergent transcription (the RNA I of about 100 bases is involved in the regulation of pColA replication and its 3'-end would be located at position 2744 according to Morlon *et al* (4)). On the other hand, we find presumptive evidence for rho-dependent termination at T2A. First, two possible specific sequence elements, found in other rho-dependent terminators, are detected ("elements I and III" according to Morgan *et al.* (17) underlined in **fig.3**). Secondly, between the *cal* coding sequence and T2Ac (position 2690 to 2759), seven C nucleotides with a spacing of 11 bases (three times) and 12 bases (three times), have been observed in an untranslated mRNA region poor in secondary structure. Such a situation has been proposed for extragenic rho-dependent termination where cytidine bases would be involved in rho binding (20).

The results for the *caa-cal* operon can be compared to those obtained for the *ceaA-kil* operon of plasmid ColE1 (22) which is very similar with respect to the functions and organization of its genes, but different in termination. Downstream from the colicin E1 gene, a rho-dependent terminator was demonstrated *in vitro* and the *ceaA-kil* operon transcription terminator was shown to be independent of rho factor (23). More *in vitro* experiments with the rho protein may confirm the effect of this protein on the termination in the *caa-cal* operon.

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