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In vivo and *in vitro* identity of site specific cleavages in the 5′ non-coding region of *ompA* and *bla* mRNA in *Escherichia coli*

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The *bla* and *ompA* gene transcripts were used as substrates to probe *Escherichia coli* extracts for ribonucleolytic activities. A site specific endoribonucleolytic activity was identified that cleaves *ompA* and *bla* mRNA. The cleavages occur *in vitro* and *in vivo*. For both the *bla* and *ompA* mRNA most of the cleavage sites which were identified map in the 5′ non-coding region. The cleavages of the *ompA* transcript have been previously suggested to regulate the growth rate dependent stability of this mRNA. Thus we propose that the identified endoribonucleolytic activity may be involved in the degradation of mRNA. Analysis of mutants revealed that the cleavages are mediated by endonucleases which do not seem to be identical to RNase III, RNase E or RNase P.

Key words: endoribonuclease/mRNA degradation *in vitro*

Introduction

Post-transcriptional regulation is important in the control of gene expression. It involves changes in mRNA, like splicing, sizing and degradation. Site specific endoribonucleases seem to play a major role in these mechanisms. In *Escherichia coli* a number of endoribonucleases have been identified which are responsible for trimming and maturation of stable RNA (Deutscher, 1985), but very little is known about enzymes controlling the processing and degradation of mRNA. However, endonucleolytic cleavage sites have been found in the intercistronic region of some polycistronic transcripts (Dunn and Studier, 1973; Guarneros *et al.*, 1982; Burton *et al.*, 1983) and for some messengers it has been shown that RNase III can catalyze such cleavages (Dunn and Studier, 1973; Guarneros *et al.*, 1982; Portier *et al.*, 1987). In bacteriophage lambda infected cells, for example, RNase III seems to control the level of *int* mRNA by cleaving in a hair-pin structure (Guarneros *et al.*, 1982). The decay of the polynucleotide phosphorylase (*pnp*) mRNA seems to be triggered by the same enzyme (Portier *et al.*, 1987; Takata *et al.*, 1987).

Determinants of mRNA stability are potential targets of endoribonucleases controlling the 'rate limiting step' of mRNA degradation. Such examples are the 5′ non-coding regions of the T4-specific *gene32* transcript and the *ompA* mRNA (Gorski *et al.*, 1985; Belasco *et al.*, 1986) that have been shown to stabilize mRNA fused downstream. In *E.coli* the stabliity of the *ompA* mRNA is related to the growth rate (Nilsson *et al.*, 1984) and we have recently identified endoribonucleolytic attacks in the 5′ non-coding region of the *ompA* transcript (Melefors and von Gabain, 1988). A correlation between rate of degradation and the rate at which the initial cleavage products appear has been described (Melefors and von Gabain, 1988). These endoribonucleolytic cleavages may therefore be involved in the regulation of *ompA* gene expression.

In order to identify and catalogue endoribonucleolytic activities in *E.coli* which might be involved in the degradation of mRNA, two different *E.coli* messengers were exposed to crude cellular extracts and assayed for cleavage products. The study was based on two monocistronic transcripts, encoding the transposon *3* derived β-lactamase protein (*bla*) and the outer membrane protein A (*ompA*). The stability and decay pattern of these messengers are different; *ompA* is a very stable mRNA while *bla* decays with a half-life which is more typical for other *E.coli* transcripts (von

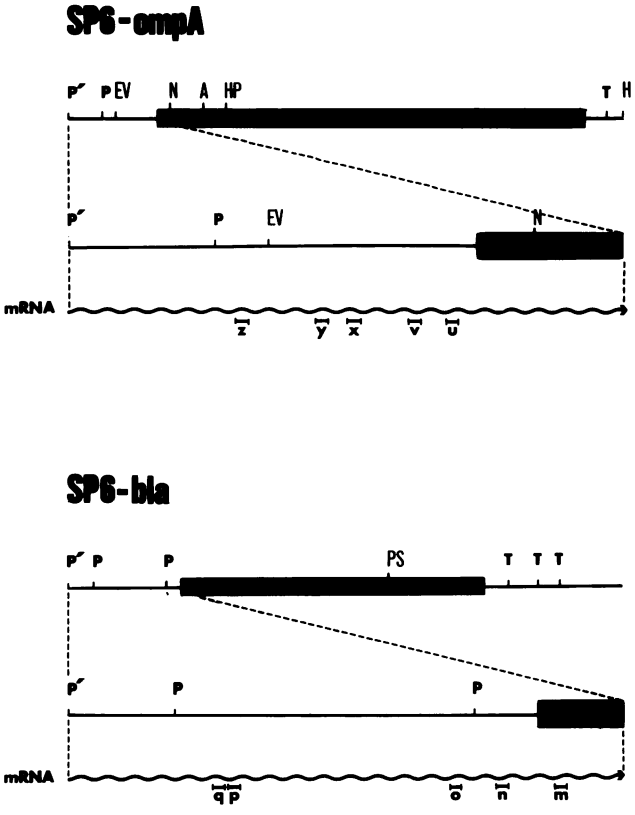

Fig. 1. Genetic maps. (**Upper**) *ompA* gene *in vitro* transcriptional unit and the 5′ part of the *ompA* mRNA. (**Lower**) *bla* gene *in vitro* transcriptional unit and the 5′ part of the *bla* mRNA. Cleavage sites are indicated, u−z and m−q and are given by the 3′ endpoints of the upstream fragments. P′, SP6 promoter; P, promoter (used *in vivo*); T, terminator; EV, *Eco*RV; N, *Nru*I; A, *Ava*II; HP, *Hpa*I; H, *Hin*dIII; PS, *Pst*I restriction endonuclease cleavage site.

Gabain *et al.*, 1983; Nilsson *et al.*, 1984). In both the messengers we investigated, specific endonucleolytic cleavages mediated by *E.coli* crude extracts were identified. The cleavages were analyzed for *in vivo* counterparts and the sites of cleavage were mapped. The responsible activity (enzyme) might reflect a class of endoribonucleases which have not yet been discovered to be involved in mRNA degradation. (For the sake of simplicity we will use activity/enzyme in the singular form when discussing our results in the following text.)

Results

An in vitro system to detect specific degradation products

The aim of the present study was to single out ribonucleolytic activities which control the initial step of mRNA degradation, from numerous exo- and endonucleases previously identified in *E.coli* extracts (Deutscher, 1985). The experimental system was based on *in vitro* transcribed radiolabeled mRNAs which were submitted to crude cellular *E.coli* extracts and assayed for specific products. Ribosomes were not present in the assay system. In Figure 1 (upper), a physical map of the *ompA* gene is shown. The *in vitro* full length transcript was initiated at the SP6 promoter and terminated by 'run off' of RNA polymerase downstream of the natural terminator at the indicated *Hin*dIII site. Thus the *in vitro* transcript is identical with the *in vivo ompA* message with the exception of 57 5′- and ~60 3′- additional nucleotides.

In the initial experiments, the full length *ompA* transcript was incubated with cellular extracts as described in Materials and methods and aliquots were withdrawn and analyzed by denaturing PAGE. While it was apparent that the intact RNA probe was decaying over the monitored time period, no cleavage products were detected that would disclose any specific mechanisms involved in the degradation (data not shown).

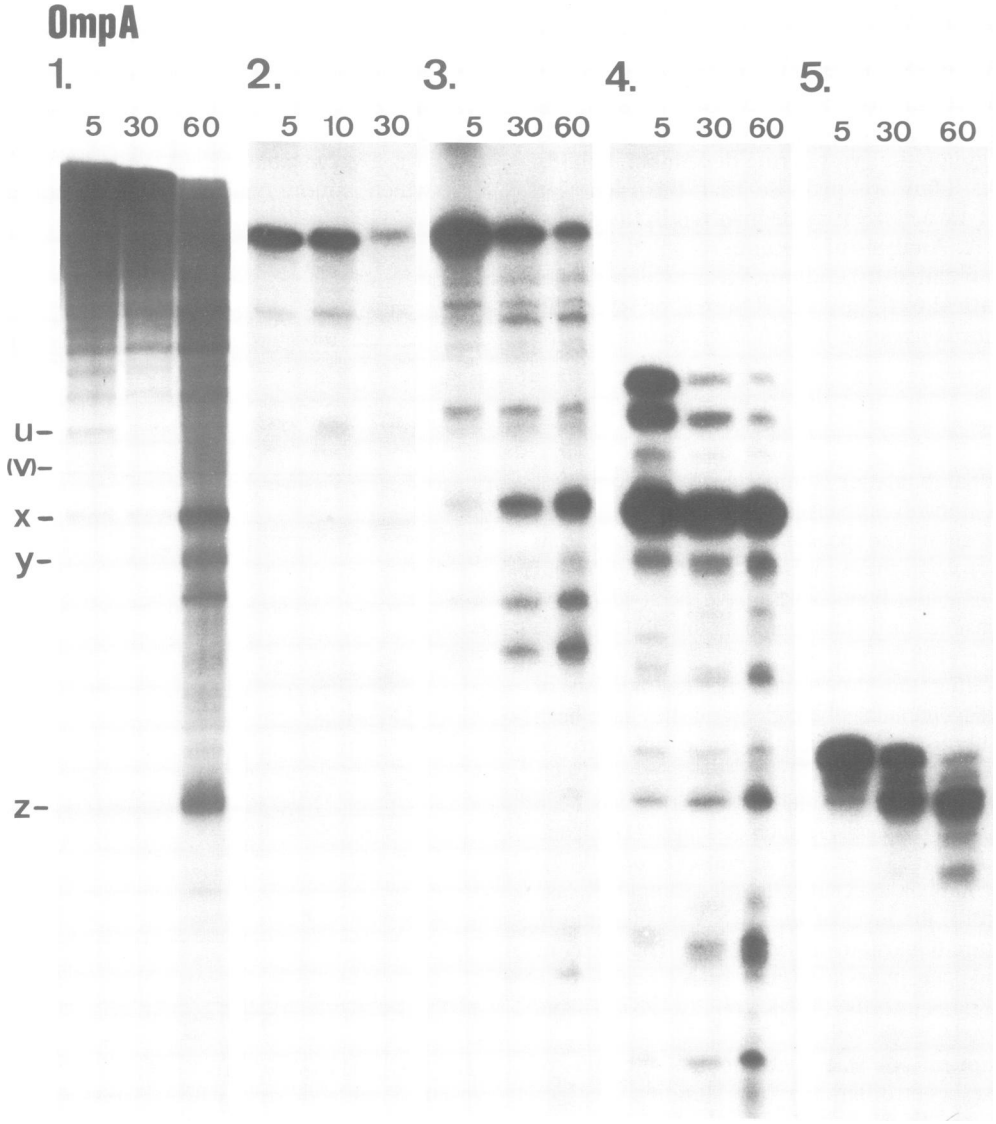

Fig. 2. *In vitro* decay of internally labeled *ompA* SP6 transcripts. Samples were taken after 5, 30 and 60 min of incubation with *E.coli* crude extracts. **Lanes 1, 3, 4** and **5** are run off transcripts from the *ompA*-plasmid linearized with *Hin*dIII, *Hpa*I, *Nru*I and *Eco*RV, respectively. **Lane 2**, *Hpa*I run off transcripts assayed in the absence of tRNA. Specific degradational products are indicated u–z. (v) indicates that v is best visible in lane 4.

In order to compete out 3′ to 5′ operating exonucleases (Deutscher, 1985) and endonucleases of low specificity, the experiments were repeated in the presence of yeast tRNA. A number of specific degradation products were now visible (Figure 2, lane 1), the concentration of some of them even increased when the degradation of the full length mRNA was followed for a prolonged time period. The degradational products were designated u, v, x, y and z.

Suitable restriction sites in the *ompA* gene (*Hpa*I, *Nru*I and *Eco*RI) made it possible to synthesize a set of *ompA* messengers which were increasingly truncated at their 3′-end (Figure 1). These mRNA derivatives were similarly used to probe the ribonucleolytic activities that were identified for the full length *ompA* mRNA. Figure 2 (lanes 3–5) shows that degradational products of the same size were visible and that such products were more pronounced when shorter templates were assayed. For the truncated template terminating at the restriction site *Eco*RV (Figure 2, lane 5) only one major degradational product was observed. Figure 2, lane 2 shows the 'run off' transcripts terminated at the *Hpa*I site assayed in the absence of tRNA and as expected, no accumulation of the degradational products was found.

Characterization of ompA degradational products

The identical size of the degradational products u, v, x, y and z, which was found for all truncated *ompA* messengers indicated that the accumulating decay intermediates originate from the 5′ non-coding region of the *ompA* mRNA (Figures 1 and 2). Therefore, *ompA* templates labeled at the 5′-end were used in the decay assay (Materials and methods). The same major degradational products u, v, x, y and z, were also detected when the 5′-labeled *ompA* transcript was submitted to the *E.coli* extracts (Figure 3A). Thus all the major degradational products share the 5′-end with the full

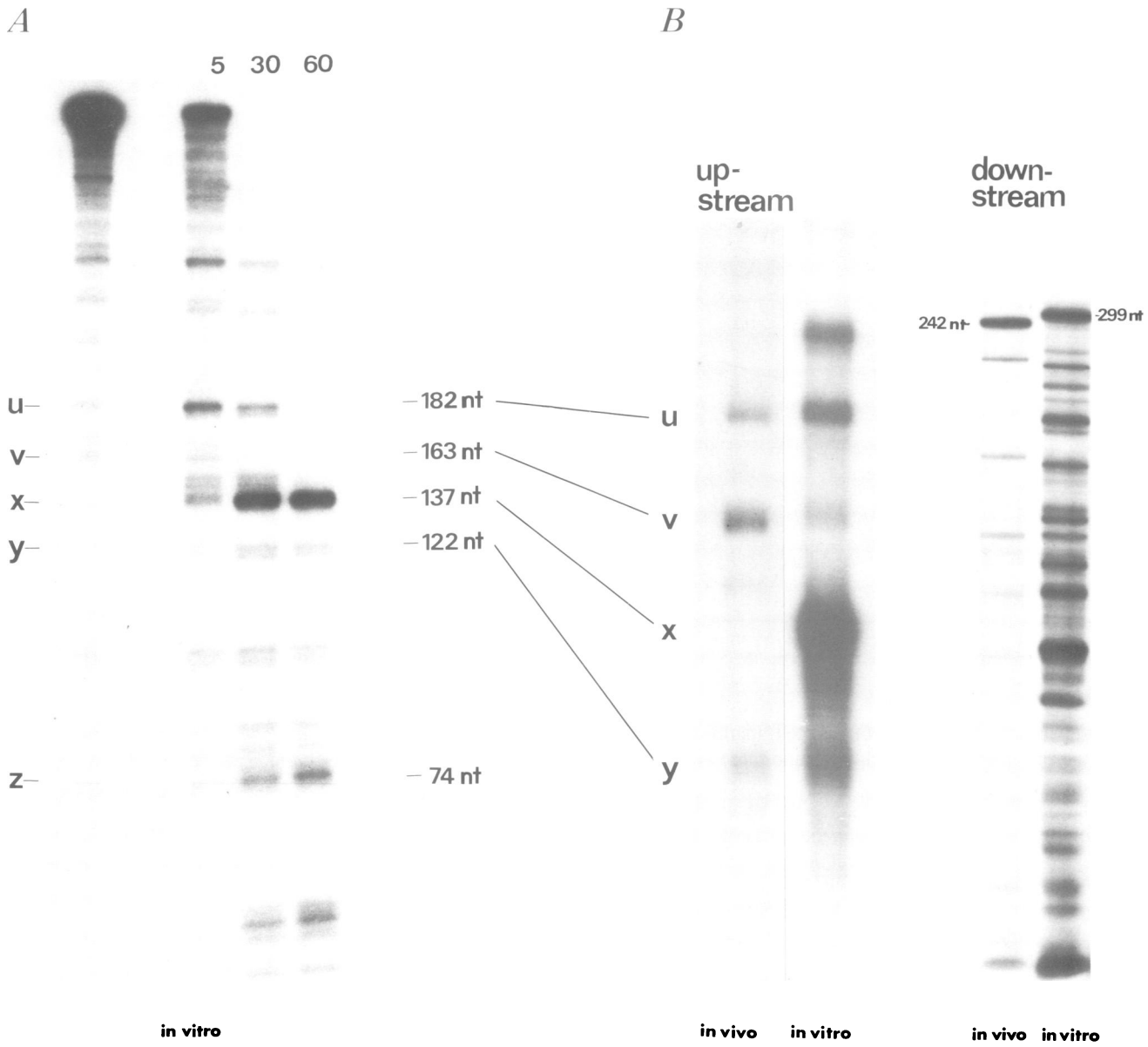

Fig. 3. Decay products of *ompA* mRNA. (**A**) *In vitro* decay of [γ-^{32}P]GTP labeled full length SP6 transcripts 5, 30 and 60 min after incubation with *E.coli* crude extracts. Size estimations were performed by semi-log plotting of size versus migration and found to be: (u)182 nt, (v)163 nt, (x)137 nt, (y)122 nt and (z)74 nt. (**B**) Up- and downstream decay products assayed *in vivo* and *in vitro*. The 242 nt and 299 nt DNA fragments correspond to the intact wild-type and SP6 transcripts respectively.

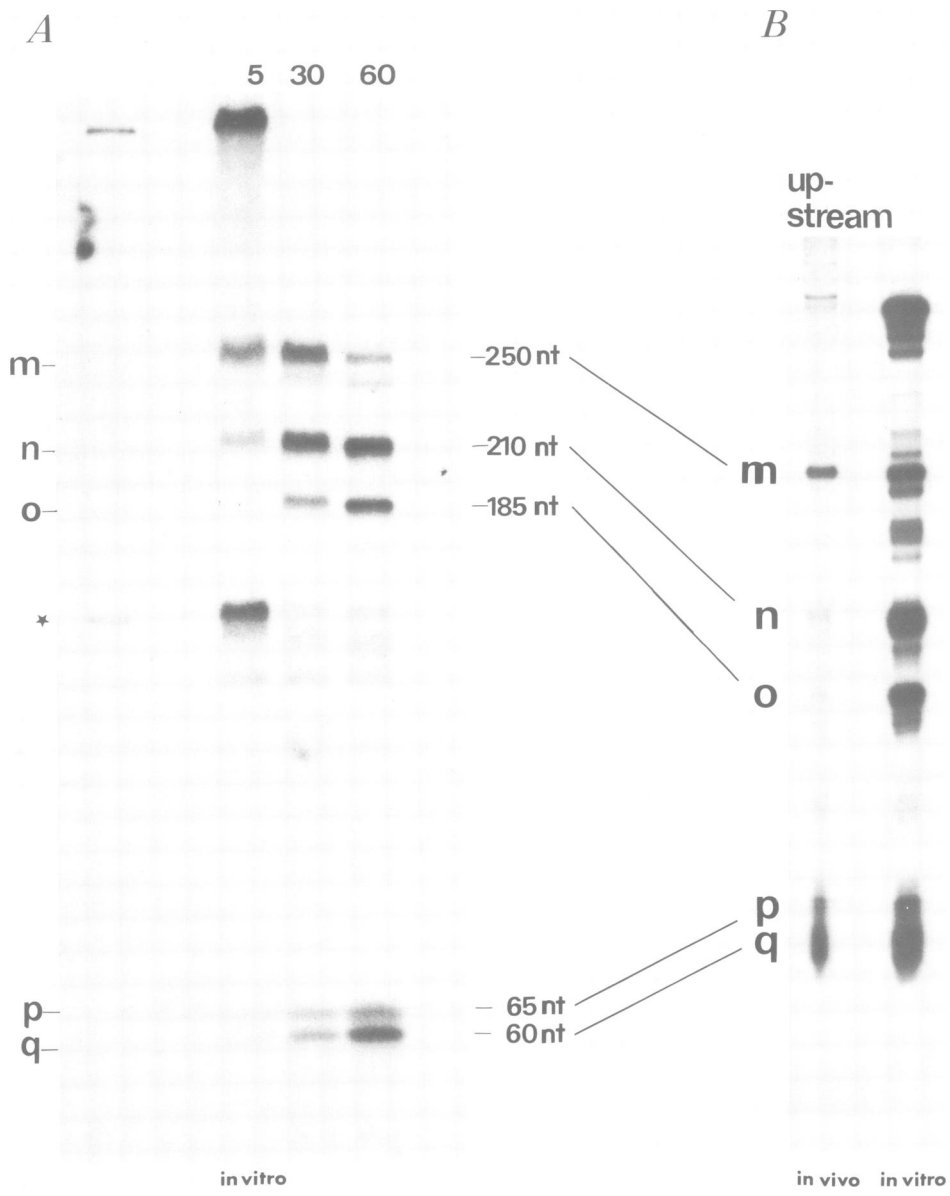

Fig. 4. Decay products of *bla* mRNA. (**A**) *In vitro* decay of [γ-^{32}P]GTP labeled *Pst*I 'run off' SP6 transcripts 5, 30 and 60 min after incubation with *E.coli* crude extracts. Specific decay products are indicated m–q. Size estimations were performed by semi-log plotting of size versus migration and found to be: (m)250 nt, (n)210 nt, (o)185 nt, (p)65 nt and (q)60 nt. *, a shorter product of 145 nt in length was always obtained from SP6 *in vitro* transcription of the *bla* gene. (**B**) Upstream products assayed *in vivo* and *in vitro*.

length transcript. The sizes of the degradational products were used to map their 3′-endpoints and these are indicated in Figure 1 (upper).

If the degradational products are the result of an endonucleolytic cleavage, it should be possible to identify cleavage products that extend from the cleavage site towards the 3′-end of the mRNA substrate (downstream cleavage product). In order to do this, *ompA* RNA submitted to a limited degradation by *E.coli* extracts was annealed with a synthetic DNA primer complementary to the *ompA* mRNA at the *Ava*II site (Figure 2, upper) and cDNA was synthesized using reverse transcriptase. In the electrophoretic analysis, a DNA product was identified that corresponds to the intact SP6 transcript. Furthermore, numerous shorter DNA products were found that proves the existence of downstream cleavage products (Figure 3B, right). The DNA fragments that would match exactly the positions of the expected cleavage sites represented only a small fraction of the downstream cleavage products. However, the DNA products obtained by reverse transcriptase show that the degradational products in u, v, x, y and z are the result of endonucleolytic activities. On the other hand the extreme diversification of the downstream cleavage products points to further ribonucleolytic activities being involved in the *in vitro* decaying system.

Probing for degradational products of ompA RNA in vivo

A recent analysis of the *ompA* mRNA *in vivo* has disclosed cleavages residing predominantly in the 5′ non-coding region of the transcript that may well reflect the rate limiting step in the growth rate dependent mRNA stability (Melefors and von Gabain, 1988; Nilsson *et al.*, 1984). The detection of such cleavages has been facilitated by over-producing the

Table I. *In vitro* biochemical characteristics of the described activity(ies)

Source of extract	Change in protocol	Effect on activity
Wild type	Mg^{2+} omitted	Activity lost
	tRNA omitted	No cleavage products
	ssDNA added	Delayed degradation
	10 mM spermidine added	No effect
	Proteinase K treatment	Activity lost
	Micrococcal nuclease treatment	No effect
	Pellet of 40% AmSP	Activity lost
	Pellet of 40–50% AmSP	Normal activity
	Supernatant of 55% AmSP	Activity lost
RNase III negative		No effect
RNase E negative		No effect
RNase P negative		No effect
RNase II negative		No effect
PNP negative		No effect

E.coli crude extracts (see Materials and methods) were used and the substrate was SP6 *ompA* mRNA (*Nru*I run off) for all conditions tested. Ammonium sulfate precipitation (AmSP): the pellets of the 40% of saturation and 40–55% of saturation precipitations were dissolved in buffer A and dialyzed against this buffer. Subsequently, the dialyzed pellets and the 55% supernatant were tested for cleavage of the substrate.

mRNA from the inducible *tac*-promoter for a short period of time. Upon over-production, cleavage products have been identified that extend from the 5′-end towards the cleavage site (upstream cleavage products) and from the cleavage products towards the 3′-end (downstream cleavage products) (Melefors and von Gabain, 1988). The *in vivo* RNA preparations were compared to the *in vitro* RNA preparation and each of the two RNA preparations was annealed to a 3′-labeled DNA probe fragment that extended from the 5′ region of both *in vivo* and *in vitro* messengers towards the coding region (Materials and methods). After treatment with S1 endonuclease, the resistant probe fragments obtained from both RNA preparations were analyzed on the same sequencing gel. Four clusters of S1-resistant DNA probe fragments were identified for both *in vivo* and *in vitro ompA* mRNA, indicating identical 5′-ends (Figure 3B, left). An extra cluster of S1-resistant DNA probe fragments was found that seems to be specific for the *in vitro* preparation (x). Additionally, both preparations were also submitted in parallel to primer extensions by reverse transcriptase as described above. Figure 3B (right) shows that identical reverse transcriptase fragments were found for both *in vivo* and *in vitro* RNA preparations. However, it should be stated that the downstream cleavage products are more pronounced for the *in vivo* RNA, while the downstream cleavage products were more diversified for the *in vitro* RNA. The results indicate that the site specific endoribonucleolytic cleavages are the same *in vivo* and *in vitro*.

Characterization of bla mRNA degradational products in vivo and in vitro

In order to extend the finding to another mRNA species, the *bla* mRNA was probed for similar degradational products. The 5′-labeled *bla* transcript was synthesized *in vitro* from the SP6-*bla* plasmid (Materials and methods). At the 3′-end it was terminated at the *Pst*I site by 'run off' (Figure 1, lower). The synthetic RNA was submitted to *E.coli* extracts as described for the *ompA* mRNA. Five degradational products m, n, o, p and q, sharing the 5′-end with the template were identified (Figure 4A). The 3′-end of four (n, o, p and q) mapped in the 5′ non-coding region of the *bla* transcripts (Figure 1, lower). Only the 3′-end of one degradational product (m) mapped in the coding region of the *bla* gene. It should be noted that the 3′-end of two degradational products (p and q) mapped in the 5′ region which is specific for the longer *bla* transcript, initiated at the reverse reading *tet* promoter (Stueber and Bujard, 1981; Brosius *et al.*, 1982).

In the same manner as described for the *ompA* mRNA, *bla* mRNA was overproduced *in vivo* by IPTG induction of the *tac*-promoter (the inducible *bla* transcript was identical to the *in vitro* transcript up to the *Eco*RI site at the reverse reading *tet* promoter) and cleavage products were compared *in vivo* and *in vitro*. When the *in vivo* RNA preparations were probed for degradational products spanning from the 5′-end to putative cleavage sites, S1-resistant DNA fragments were identified that match the 3′-ends of the *in vitro* degradational products (Figure 4B). Thus the *bla* mRNA disclosed degradational products that were identical *in vivo* and *in vitro*, and the cleavage sites mapped in the 5′ non-coding region similarly to the *ompA* mRNA.

Characterization of the degradational activity

The *in vitro* degradation of the *ompA* and *bla* mRNA was dependent on the *E.coli* extracts. A number of experiments were performed to characterize further the ribonucleolytic activity that is mediated by the extracts. *ompA* mRNA was not found to be degraded to specific products when the extracts were pre-incubated with proteinase K (Table I). Pre-incubation with micrococcal nuclease, and subsequent inactivation of this enzyme by EGTA, did not affect the degrading activity (Table I). The activity was tested in the absence and presence (100 mM) of K^+ ions and no significant difference was found (Table I). However, omitting Mg^{2+} ions caused a total loss of the ribonucleolytic activity. Thus the identified activity seems to belong to a class of ribonucleases which are Mg^{2+} dependent. When the reaction mixture was supplemented with 10 mM spermidine or with single stranded DNA (10–250 ng/assay volume) the reaction was slightly retarded, especially if the extract was pre-incubated with these substances prior to template addition (Table I). Extracts were also prepared from *E.coli* mutants lacking certain RNase activities. A pair of isogenic strains respectively lacking and exhibiting RNase III activity was found to degrade *ompA* mRNA with the same specificity as the *E.coli* strain C600 (Table I). Extracts from an isogenic pair of strains, one reported to be temperature sensitive for RNase E, were compared and no differences in degradational products were found. Similar experiments with strains that were defective for RNase II or polynucleotide phosphorylase (PNP) disclosed that neither of the two 3′ to 5′ operating exonucleases in itself can explain the identified degradational products (Table I). A mutant lacking RNase P activity revealed the same activity as were found for the *E.coli* strain C600 (Table I).

A first step towards purification of a protein with such an enzymatic activity is the fractionation of *E.coli* extracts using ammonium sulfate. Two precipitates obtained by adding ammonium sulfate to 40% of saturation and then increasing to 55% of saturation at 4°C were assayed for the

cleavage activity as well as supernatant from the 55% precipitation. The activity was only identified in the precipitate obtained by increasing the ammonium sulfate concentration from 40 to 55% of saturation (Table I); the cleavage products were shown (by co-migration) to be identical to those which were observed when the total *E.coli* extracts were analyzed. The results show that the cleavage activity can be recovered within a relatively narrow range of ammonium sulfate fractionation.

Discussion

We have identified an enzymatic activity in *E.coli* extracts that is capable of processing the *bla* and *ompA* mRNA species to specific degradational products. Most of them were identical to endonucleolytic cleavage products which were also identified for the homologous mRNA species *in vivo*. For the *ompA* mRNA, these cleavages have been shown to be involved in the growth rate dependent degradation (Melefors and von Gabain, 1988). The identified attack sites predominantly map in the 5′ non-coding region of the two mRNA species, and they may well inactivate the mRNA, e.g. by interfering with the ribosomal loading. We propose that the enzymatic activity which is not identical to RNase III or RNase E, is important for the rate limiting step(s) controlling the degradation of the mRNA species investigated.

The endonucleolytic activity identified in vitro

In order to assay for enzymatic activities that might be involved in the degradation of mRNA, the fate of two mRNA species was studied in an *in vitro* system. To simplify the analysis, ribosomes were omitted in the assay system.

Our experiments disclosed the existence of distinct RNA breakdown products. The accumulating RNA products constituted RNA segments ranging from the native 5′-end of the mRNA to the putative attacking site. The endonucleolytic nature of the discovered activity was verified by showing the existence of cleavage products that extend downstream of the putative cleavage site. However, the fragment pattern of the downstream products was much more diversified. This result could be explained by multiple endonucleolytic attacks over a sensitive region or by a processive degradation towards the 3′-end that follows the initial attack.

The accumulation of the upstream cleavage products in the *in vitro* situation shows that the 3′-termini must be relatively resistant to further degradation. On the other hand, the appearance of distinct degradational products was entirely dependent on the presence of tRNA and it is likely that the competitive RNA is capable of titrating out the 3′ to 5′ operating exoribonucleases. At the same time, the competitor seemed not to inhibit the endonucleolytic activities.

The present experiments demonstrate the site-specific nature of the endonucleolytic activity. It remains to be demonstrated whether the specificity resides in the primary sequences of the probed RNAs, in the spatial structure or in a combination of both. The analysis of various truncated *ompA* mRNAs allows us to conclude that the specificity of the cleavages may reside within a region as small as 100 nt, which contains the majority of the attack sites. Hence, *in vitro* a long distance folding of the RNA molecule seems to be unimportant for the occurrence of cleavages.

In vivo and in vitro identity of the cleavages

Most of the upstream cleavage products, and some of the downstream cleavage products, were found to be identical *in vivo* and *in vitro* as indicated by co-migration. However, the *in vitro* result departs from the *in vivo* situation in that the upstream cleavage products were more abundant. Furthermore, the downstream cleavage products were more diversified *in vitro* than *in vivo*. Such differences might be explained by different kinetics of the secondary events in the degradation process. The lack of ribosomes, and the presence of tRNA competitor, might alter the stability of the intermediary breakdown products. *In vivo*, upstream cleavage products are more likely to be rapidly degraded by 3′ to 5′ exoribonucleases, while ribosomes might retard the secondary degradation of the downstream cleavage products.

The identified activity is involved in the degradation of the ompA and bla mRNA species

The biological importance of the identified cleavages has not yet been elucidated. We propose that the cleavages control the initial steps of degradation. Such an interpretation rests on three lines of argument. For some of the cleavages in the 5′ non-coding region of the *ompA* message, it has been observed that the stability of the mRNA follows the rate at which these cleavages occur (Melefors and von Gabain, 1988). Cleavages in 5′ non-coding region of both the *ompA* and *bla* mRNAs are compatible with the finding that this part of the transcript might control their stability (Belasco *et al.*, 1986; Nilsson *et al.*, 1987). Finally, the lack of cleavages in the coding region of the *bla* mRNA (down to the *Pst*I site) agrees well with the recent observation that no extra cleavages have been identified when most of the coding region is deprived of ribosomes (Nilsson *et al.*, 1987).

The exact mechanisms by which endonucleolytic attacks might initiate the degradation of mRNA remains to be explored. Endonucleolytic activities targeting the 5′ non-coding regions might either open the message to a wave of further attacks that degrade the mRNA towards the 3′-end or the activity might interfere with the loading of ribosomes. The latter interpretation is supported by the observation that the complete absence of ribosomes on a transcript causes a shorter half-life (Nilsson *et al.*, 1987).

Characterization of the identified activity

The identified activity is likely to be a protein. At present the possibility cannot be excluded that the activity reflects more than one enzyme and it is not certain whether the cleavages found for the *bla* and the *ompA* messages were catalyzed by the same enzyme. However, the results of the ammonium sulfate fractionation may be interpreted in such a way that the activity is defined by a rather limited number of enzymes. On the other hand, experiments performed on the *ompA* mRNA suggest that the enzyme is not identical to RNase III, RNase E or RNase P. The specificity of the cleavage pattern does not change if the analyzed extracts are derived from strains lacking either RNase II or PNP. However, these two 3′ to 5′ exoribonucleases can replace each other *in vivo*. Therefore, the cleavage products may be independent of these two enzymes. Finally, the Mg^{2+} dependence of the cleavage region allows us to exclude that

the activity is identical to, for example, RNase I, RNase N or RNase F.

While the present results are not sufficient to show whether the enzyme is identical to any other of the previously characterized RNases, it is evident that such substrate specificity has not been reported before. The novel specificity makes the enzyme a good candidate for a class of mRNases that are important in controlling the level of mRNA in the cell.

Materials and methods

Bacterial strains

E.coli strains employed in these studies included C600, SK2299 (RNase II$^-$) and SK694 (PNP$^-$) which were kindly provided by S.Kushner. A49ts (RNase P), N3431 (RNase E$^-$ ts) and BL321 (RNase III$^-$) were generously given to us by Drs S.Altman, M.Belfort and W.Studier, respectively.

Plasmids

Vectors pSP65 and pGEM4 were from Promega. The *ompA* gene containing an amber codon at position 31 was excised from plasmid pTU500 (Freundl *et al.*, 1985) and inserted into plasmid pPS65 using the *Pst*I site of both plasmids. The insert included the native transcriptional stop signal. For the *bla* gene an insert ranging from the *Eco*RI to the *Pst*I site from pBR322 was ligated into pGEM4. The resulting *in vitro* transcript contained almost the complete 5′-end of the longer *in vivo* mRNA but was lacking the last 30% of the coding part and the transcriptional stop signal. Both plasmids were constructed so that the sense mRNA was transcribed when SP6 RNA-polymerase was used. Plasmid pMMB66-*ompA* has the *ompA* gene under the control of the inducible *tac*-promoter and was kindly provided by Ö.Melefors. A similar construct, pMMB66-*bla*, was made by isolating the 460 bp *Bcl*I−*Pvu*I fragment from pJB322 (Belasco *et al.*, 1986) containing a part of the *bla* gene. The fragment was ligated to *Bam*HI−*Pvu*I digested pMMB66 and the resultant β-lactamase product was confirmed by minicell analysis.

In vitro transcription

The various templates were linearized with the desired restriction endonuclease and tested by agarose gel electrophoresis for complete digestion. The linearized DNA (1−5 μg) was transcribed in a 50 μl reaction volume containing 40 mM Tris−HCl, pH 7.5, 6 mM MgCl, 2 mM spermidine, 50 U RNasine (Promega), 10 mM DTT, 300 μM rNTPs and 0.5 μM [α-^{32}P]CTP (Amersham). The reaction was started by addition of 1 U SP6 RNA-polymerase (Boehringer) to the reaction mixture, followed by 1 h of incubation at 37°C. The DNA template was removed by addition of RNase free DNAse I, and after 10 min the reaction was terminated by extraction with an equal volume of phenol and purified over a G50-sephadex column. RNA was labeled at the 5′-end by transcribing the template in the presence of 100 μM [γ-^{32}P]GTP for 1 min before addition of 300 μM 'cold' rNTPs. The reaction was further processed as described above.

Cell extracts

The described *E.coli* strains were grown in L-Broth (200 ml) to a cell density of OD_{600} = 0.5 and harvested by centrifugation at 5000 r.p.m. for 5 min. The cells were washed once and the pellet was resuspended in 1.5 ml Buffer A (10 mM Tris−HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM DTT, 5% glycerol), 0.45 mg lysozyme was added. Cells were mildly lysed by alternating freezing and thawings of the suspension. Thereafter, 3 ml of Buffer B (Buffer A + 1 M KCl) was added, mixed on ice for 5 min and centrifuged for 2 h at 105 000 *g*. The supernatant was frozen in aliquots until further use. Ammonium sulfate precipitations were done as described by Dunn (1976).

The extracts from a pair of isogenic strains lacking or exhibiting RNase III activity, was first tested on *in vitro* transcribed early T7 mRNA and the genotype of both was confirmed.

Degradation assay

Standard reaction (50 μl) contained assay buffer (20 mM Tris−HCl, pH 7.9, 10 mM MgCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol), tRNA (0.5 μg/μl) and 125−250 ng of the *in vitro* transcript. An appropriate amount of *E.coli* cell extract (usually 5 μl) was then added and the reaction mixture was incubated at 30°C. At different time intervals aliquots were collected in equal volume of phenol containing 10 mM EDTA. After phenol extraction the samples were ethanol precipitated and analyzed by 6% PAGE.

Analysis of decay products in vivo and in vitro

Total RNA from C600 cells containing pMMB66-*ompA* or pMMB66-*bla* plasmids was prepared 15 min after IPTG (1 mM) induction; RNA isolation was as described earlier (von Gabain *et al.*, 1983). *In vitro* RNA was prepared as described above. RNA products extending from the putative endonucleolytic attack site were analyzed by the following DNA probes: the plasmid pMMB66-*ompA* was linearized at the *Eco*RI site and 5′-labeled using T4 DNA polymerase or the plasmid pBR322 was linearized at the *Aat*II site and 5′-labeled using terminal transferase. Hybridization and S1 protection experiments were carried out as described (von Gabain *et al.*, 1983). For analysis of RNA products extending downstream of the putative cleavage site an oligonucleotide of 24 nt homologus to the *ompA* transcript was used for reverse transcription (Melefors and von Gabain, 1988).

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