A stable core region of the tra operon mRNA of plasmid R1-19

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Received January 5, 1989; Accepted January 23, 1989 EMBL accession no. X13681

ABSTRACT
The degradation of the polycistronic tra-mRNA of the resistance plasmid R1-19 leads to the accumulation of a well defined series of stable mRNA species. The majority of the most stable mRNAs contains the message for the traA gene only. The differently sized stable mRNAs possess a common 3' terminus within the traL gene but vary at their 5' ends. The 3' terminus probably results from protection against exoribonucleases by a secondary structural feature. We propose that the 5' ends are generated by endoribonucleolytic cleavage. The stability of this part of the tra-mRNA exceeds 30 minutes and probably increases the rate of expression of the traA gene product propilin, the precursor of the sex pilus subunit. The expression of propilin and its processing into a protein of the molecular weight of mature pilin is demonstrated with the isolated gene. The sequence of the so far unknown genes traL and traE of R1-19 is presented.

INTRODUCTION
It has become increasingly evident in the last years that the stability of mRNA in prokaryotic organisms represents a major factor in the efficiency of gene expression (1). Differential mRNA stability may be as important as transcriptional or translational control. Bacterial mRNA stabilities vary greatly; generally the half-life appears to be in the order of a few minutes. A number of mRNAs, however, are considerably more stable, like the mRNA for OmpA, the major outer membrane protein of E.coli. The stability of the monocistronic ompA message was found to be growth-rate dependent (2). Other examples have been reported showing that certain segments in a polycistronic mRNA decay significantly slower than the rest of the molecule like the 3' portion of the puf transcript of Rhodobacter capsulatus which is rapidly degraded to give rise to either of two slowly decaying mRNA remnants (3). In the case of the papA mRNA within the pap pili gene cluster a primary transcript also encoding the papB regulatory gene product is endonucleolytically cleaved. The cleavage results in the rapid decay of the papB encoding 5’ half of the mRNA, whereas the papA encoding 3’ half remains as a stable transcript. In this way mRNAs coding for the major pilus subunit accumulate (4). Another example of differential mRNA stability is the polycistronic malEFG operon of E.coli (5).

In E.coli two enzymes are primarily responsible for the bulk degradation of mRNA, RNase II and polynucleotide phosphorylase (5). These enzymes are 3'–5' exoribonucleases. Special secondary structures within or at the end of a mRNA are known to protect the upstream lying message against the degrading activity of the 3'–5' exoribonucleases (1). No 5'-3' exoribonuclease has yet been identified in E.coli (6). On the basis of the latest results it has been proposed that in addition to the better characterized 3' exoribonucleases
specific endoribonucleases may be functional recognizing structural features or specific sequences near the 5’ terminus of a mRNA. It has been shown that the cleavages in the 5’ regions of both the _bla_ and _ompA_ mRNAs are mediated by endoribonucleases which do not seem to be identical to RNase III, RNase E or RNase P (7). However, in the case of the processing of the primary gene 32 transcript first evidence was provided that host-encoded RNase E is essential for the processing of the mRNA (8).

Plasmids of the IncF group are known for a giant (approximately 33 kb) mRNA molecule which carries near its 5’ end the coding information for pilin, the major building block of the bacterial sex-pili. The mature pilin protein is a proteolytic product of propilin, the immediate translation product of the _traA_ message (for recent reviews see 9–11).

The large amount of pilin within cells harbouring the F plasmid (10) prompted us to investigate the stability of portions of the _tra_ operon mRNA of R1-19, a derepressed mutant of the resistance plasmid R1 (12). R1 is closely related to the F plasmid showing a high degree of homolgy in the _tra_ region (13–17). In preliminary studies a stable, approximately 400–600 nt long, mRNA isolated from R1-19 harbouring _E.coli_ cells could be shown to be coded by a 2.1kb _PstI_ DNA fragment that includes the 3’half of _traI_, the _traY-Z_ promoter and the _traYALE_ genes. We designed experiments to characterize further this stable mRNA and found stable segments which are identical with the _traA_ encoding portion of the _traYZ_ mRNA. We present evidence that the primary 32kb _tra_ operon mRNA is processed by endo- and exoribonucleolytic activities generating stable mRNA species with distinct 5’ends and a common 3’end.

**MATERIALS AND METHODS**

**Bacterial strains**

_E.coli_ K12 5K (tre, thi, _rpsl_+, _kdsR_, _kdsM_+, lac)

_E.coli_ MV1190 Δ(lac-proAB), thi, _supE44_,

Δ(srl-rec4)303::Tnl0(tet') [F':_traD36_, _proAB_, _lacFzΔM15_]

_E.coli_ K38 has been described by Tabor and Richardson (18). T7 RNA polymerase/promoter strains were obtained from the laboratory of August Bock with the permission of Stanley Tabor. _E.coli_ MV1190 was obtained from Jeff Vieira.

**Enzymes and chemicals**

Nuclease S1 was purchased from Bethesda Research Laboratories. DNA polymerase I-large fragment (Klenow fragment), Hybond M&G, universal M13 sequencing primer, T4 DNA polynucleotide kinase, [32P]-Orthophosphate (10 mCi/ml), gamma-[32P]-ATP (>5000 Ci/mmol), α[32P]-dCTP (>400 Ci/mmol) and L[35S]-methionine (>800 Ci/mmol) were purchased from Amersham International. Restriction Enzymes and T4 DNA Ligase were purchased from Boehringer Mannheim GmbH. Specific oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer.

**DNA manipulations**

Recombinant DNA techniques were performed according to Maniatis (19), or the manufacturers’ protocols. Transformations were carried out using either the standard CaCl2 protocol or a shorter version thereof (20).

**Construction of recombinant plasmids**

The construction of pEO deletion subclones is described in reference 15. The pEO subclones were characterized by restriction mapping and used for the Maxam and Gilbert sequencing method and for the Southern hybridization experiment. _pSF1212_ (15) was the source for the 2.1kb _PstI_ fragment cloned into the _PstI_ site of _pUC119_ yielding pGK101/I and...
pGK101/II, respectively. pGK subclones were obtained by exonuclease Bal31 digestions of BamHI cut pEO2. The ends were filled in with the Klenow fragment, the DNA was cleaved with PstI and directly cloned into Smal/PstI cut pUC119. The resulting subclones pGK102, pGK103, pGK105 and pGK106 were used for the Sanger dideoxy sequencing reactions and for the generation of the probes used in the nuclease SI protection assays. For the expression studies XhoI/EcoRI fragments from pGK106 (containing only traA) and pGK103 (containing both traA and traL) were cloned into BamHI/EcoRI cleaved pT7/6 (Tabor, S. personal communication). The resulting expression clones were designated pT7A and pT7AL, respectively.

Preparation of ss DNA
Single stranded DNAs were obtained using the pUC119/M13K07/MV1190 system described by Vieira and Messing (22 and Vieira, J. personal communication).

DNA sequencing
The nucleotide sequence was determined on both strands either by the method of Maxam and Gilbert (22), a modification thereof using Hybond M&G as a solid support for the DNA to be sequenced, or by dideoxy chain termination (23). Specific oligonucleotide primers were used to facilitate completion of the sequencing strategy.

DNA probes
2μg single-stranded DNA of the appropriate recombinant plasmids were annealed to 20 ng universal M13 sequencing primer in Klenow reaction buffer (final concentration:10 mM Tris pH 8.0, 5 mM MgCl₂), the DNA was polymerized in the presence of 0.05 mM d(AGT)TPs, 40 μCi α[32P]-dCTP and 5U Klenow fragment to give the double stranded form. After 45 min at 37°C 5 μl 0.5 mM of each of the dNTPs were added. The mixture was incubated for 15 min at room-temperature, the final volume of the reaction was 35 μl. The enzyme was inactivated by heating for 10 min at 70°C. Subsequently restriction enzyme digests were performed by adding the appropriate restriction enzymes, buffer and water to a final volume of 50 μl. The mixture was incubated overnight at 37°C. The appropriate DNA fragments were isolated from low melting agarose. The recombinant plasmids used, the restriction enzymes and the fragment lengths of the resulting probes are shown in table I.

RNA preparations
In vivo 32P-labelled RNA was prepared as follows: E.coli cells harbouring plasmids R1-19 or, as a control, without plasmid were grown overnight in 40 ml TY medium at 37°C. Cells were harvested by 5 min centrifugation at 4000×g and washed with 0.9% sodium chloride. After another centrifugation step the cells were resuspended in phosphate-free medium as described by Garen and Levinthal (24) and grown to A₆₀₀=0.4. 1.5 mCi

Table I. DNA PROBES

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>RESTRICTION ENZYMES</th>
<th>5'END</th>
<th>3'END</th>
<th>LENGTH</th>
<th>PROBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGK106</td>
<td>EcoRI/PstI</td>
<td>4437</td>
<td>3154</td>
<td>1283</td>
<td>A</td>
</tr>
<tr>
<td>pGK101/II</td>
<td>HincII</td>
<td>5296</td>
<td>4780</td>
<td>516</td>
<td>B</td>
</tr>
<tr>
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<td>HincII</td>
<td>4780</td>
<td>3938</td>
<td>842</td>
<td>C</td>
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<tr>
<td>pGK103</td>
<td>EcoRI/HincII</td>
<td>4680</td>
<td>3938</td>
<td>742</td>
<td>D</td>
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<tr>
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<td>EcoRI/HincII</td>
<td>4437</td>
<td>3938</td>
<td>499</td>
<td>E</td>
</tr>
<tr>
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<td>4437</td>
<td>3858</td>
<td>579</td>
<td>F</td>
</tr>
<tr>
<td>pGK106</td>
<td>HincII</td>
<td>4206</td>
<td>3938</td>
<td>268</td>
<td>G</td>
</tr>
</tbody>
</table>

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Figure 1. Physical and genetic map of the 2.1 kb PstI fragment of Rl. The genes and restriction sites are shown in panel B. P, X, H and N designate cleavage sites for PstI, XhoII, HindIII and NdeI, respectively. A: Sequencing strategy. Open arrows: chemical sequencing, filled arrows: dideoxy sequencing. C: Probes used in the S1 nuclease protection experiments. D: Fragments of deletion subclones used for the Southern hybridization experiment.

$^{32}$P-orthophosphate was added and the cells incubated to $A_{600}$ = 0.5. Rifampicin was added to give a final concentration of 100 $\mu$g/ml. Incubation was continued for 30 min. The cells were harvested and washed as described above, the pellet was resuspended in 5 ml 1 mM MgCl$_2$ solution, and the RNA extracted by addition of 10 ml water-saturated phenol. After stirring for 30 min at 4°C the aqueous phase was recovered and the RNA twice ethanol precipitated. The resulting RNA pellet was suspended in 75 $\mu$l doubly distilled water, 50 $\mu$l loading buffer (95% deionized formamide, 10 mM EDTA, 0.1% xylene cyanole FF, 0.1% bromophenol blue) was added to the RNA solution and the mixture loaded onto a 1.5×200×400 mm 8% polyacrylamide-7M urea gel. After electrophoresis for 3 hrs at 40 mA the wet gel was covered with plastic foil and autoradiographed for 15 min. The ‘large’ RNA species were identified as the top band and extracted from the gel by cutting the first 30 mm of the gel and soaking the crushed gel pieces in 5 ml 2×SSPE at 4°C overnight. Finally, 5 ml RNA solution with a concentration of 50 $\mu$g/ml was recovered. The specific activity was $1\times10^5$ cpm/$\mu$g RNA. Only RNA extracted from Rl-19 containing cells was subjected to this purification procedure, control RNA from E.coli K12 5K was used directly in the Southern hybridization experiment described below. RNA concentrations were measured, 100 $\mu$g aliquots of each sample were stored at $-80^\circ$C.

Southern Hybridization

1 $\mu$g plasmid DNA of the appropriate subclones was cleaved with PstI and the fragments (Figure 1D) were separated by agarose gel electrophoresis. The DNA was transferred onto
Figure 2. Polynucleotide sequence spanning the region from the end of \textit{traJ} to the end of \textit{traE}. The genes were translated into amino acid sequences which are represented by the one letter code. Numbering begins at the \textit{EcoRI} site left of the \textit{traJ} gene.

nitrocellulose filters, prehybridized, probed and washed essentially as described by Maniatis et al. (19). $2 \times 10^5$ cpm per square centimeter of nitrocellulose sheet of \textit{in vivo} $^{32}$P-labelled \textit{'large'} RNA from \textit{E.coli}, containing plasmid R1-19, was used as a probe. In a control

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experiment in vivo $^{32}$P-labelled RNA from E.coli K12 5K was used to probe the same DNA fragments.

**S1 nuclease protection assays**

S1 nuclease protection assays were performed according to Berk and Sharp (26). 10 or 20 µg total cellular RNA was carefully dissolved in 50 µl hybridization buffer containing 80% formamide, 20 mM PIPES and 0.4M NaCl. The 0.01 pmol DNA probe (specific activity of Cerenkov radiation was about $1 \times 10^8$ cpm/µg DNA) was added and the mixture incubated at 75°C for 15 min. DNA–RNA hybrids were allowed to form at 45°C over night. The correct hybridization temperature was determined empirically. The hybrids were treated with S1 nuclease at final concentrations of 1000 or 200 U/ml buffer containing 30 mM Na-acetate (pH 4.6), 50 mM NaCl, 1 mM ZnSO$_4$ and 5% glycerol. The protected DNA fragments were subsequently analysed on denaturing polyacrylamide-8M urea sequencing gels.

**Protein expression**

For the expression of traA and traL genes the expression system developed by Tabor and Richardson was used (18 and personal communication). Recombinant plasmids pT7A and pT7AL were transformed into the E.coli strain K38 containing pGPl-2 (18). Cells harbouring both pGPl-2 and the pT7 recombinant plasmids were grown in 2×TY medium (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter) in the presence of each 40 µg/ml ampicillin and kanamycin at 30°C. At A$_{590}$=0.8, 0.2 ml of the cell suspension was centrifuged. The cell pellet was washed with 1 ml M9 (25) medium twice and recentrifuged. The cell pellet was resuspended in 1.0 ml of M9 medium supplemented with thiamine and 18 amino acids (27) (minus cysteine and methionine). Cells were grown with shaking at 30°C for 90 min, then the temperature was shifted to 42°C followed by incubation for 15 min. Rifampicin was added to a final concentration of 200 µg/ml. After additional incubation for 10 min at 42°C the temperature was shifted down to 30°C and kept there for 20 min. Samples were pulsed with 10 µCi of [35S]-methionine for 1 min. In the chase experiment this incubation was followed by the addition of nonradioactive methionine (final concentration 0.1%). 240 µl aliquots were removed at 0, 5, 15 and 60 min of chase. Cells

![Figure 3. Expression of traA and chase of the label. Two plasmids, pT7A (track A) and pT7AL (track B) were used to express the genes carried by the inserts. Both contain DNA beginning with the XhoI site shown in figures 1 and 2. The former insert ends at nt 4437 the latter at nt 4680. In both lanes only the traA gene product (traA) and its processed version, designated traA*, could be detected. The initial pulse with radioactive methionine was followed by a chase with nonradioactive methionine for the periods indicated. An autoradiogram of an SDS-PAGE is shown.](https://academic.oup.com/nar/article-abstract/17/4/1283/1258033)
were centrifuged, the cell pellet was suspended in 30 μl cracking buffer (60 mM Tris-HC. pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue), heated to 95°C for 3 min and loaded onto a 20% polyacrylamide SDS gel (28).

RESULTS

Sequence

The nucleotide sequence of R1 from oriT up to the end of traJ has been reported previously (15–17). The sequence of R1-19 which presumably is identical with that of R1 in the segment which is relevant for this work has been described by another group just beyond the traA gene (14,29). In preliminary experiments, a stable mRNA was found to hybridize to a 2.1 kb PstI fragment comprising part of the traJ gene and the first four genes of the tra operon (Ostermann and Hogenauer unpublished). In order to be able to localize the 5'- and 3' ends of the stable mRNA we decided to sequence the still unknown part of the PstI fragment of R1.

Both the chemical and the dideoxy sequencing methods were used. For the chemical sequencing method already available deletions of pSFI220 were exploited. Their preparation was described earlier (15) and their lengths were determined by restriction mapping. The appropriate deletions were selected. They are designated with the prefix pEO, some of them are depicted in figure 1. The two sequencing runs in the middle of the traE gene in both orientations were carried out from an appropriately located HpaII site.

A series of further deletions was prepared using Bal31 exonuclease. The resulting clones are designated with the prefix pGK. These deletions originate from the rightward PstI site and were sequenced by the dideoxy method using both the universal and specific oligonucleotide primers.

The sequence is shown in figure 2 and is identical to the published R1 and R1-19 sequences (17,14,29), except for two sequencing errors at positions 889 and 1069 in our

![Figure 4. Southern hybridization experiment. Deletion subclones were cut with appropriate restriction enzymes and separated on an agarose gel. After transfer to nitrocellulose the DNA was hybridized with 32P-labelled RNA extracted 30 min after rifampicin addition from E.coli cells harbouring plasmid R1-19. Tracks 1 to 5 contain DNA of pEO2 to pEO10, respectively. All fragments represented in figure 1D, except the shortest, gave signals.](https://academic.oup.com/nar/article-abstract/17/4/1283/1258033)
earlier sequence (17). In the first case a T had to be deleted at the position indicated, in
the second an A residue was inserted.

The sequence contains four complete open reading frames (ORFs) which we, by
comparison with the homologous sequences of other IncF plasmids, assign as the \textit{traYALE}
cistrons. The sequences for the \textit{traL} and \textit{traE} genes are new. Comparison with the sequence
of the F plasmid shows 99\% identity for \textit{traL} and 98\% for \textit{traE} at the nucleotide level,
the only difference between the two \textit{traL} genes being the occurrence of a second methionine
codon at the beginning of the gene in R1. There is no ribosome binding site (RBS) in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Nuclease SI protection experiment showing the kinetics of \textit{tra} mRNA degradation. Total RNA from \textit{E. coli} K12 5K containing plasmid R1-19 was isolated at the indicated incubation periods after rifampicin
treatment. The RNA was hybridized with uniformly radiolabelled DNA fragments 'A' and 'B' (Figure 1),
subjected to treatment with 200 U/ml SI nuclease and fractionated by polyacrylamide gel electrophoresis. \textit{HaeIII}
(M1) and \textit{HpaII} (M2) digests of pUC118 DNA served as molecular size standards. An autoradiogram of the
gel is shown. Arrows denote the most prominent protected fragments.}
\end{figure}
the intercistronic region between *traA* and *traL*. As a consequence of two frame shifts within *traE* we find a lower identity of 92% at the amino acid level in the two *traE* genes of R1 and F.

**Expression of traA**

In order to show that the DNA region which codes for a stable mRNA is indeed translated into a protein of the expected size we performed expression studies using the pT7 system (18 and personal communication). This system was chosen because fragments containing only the *traA* gene lack their own promoter and the pT7 plasmids provide a strong foreign promoter recognized by T7 polymerase only. Fragments encompassing either the complete *traA* gene or the *traA* and *traL* genes were placed downstream the T7 promoter of the expression vectors pT7/5 or pT7/6. Recombinant plasmids were transformed into *E.coli* K38 [pGP1], a strain which contains the gene for the T7 RNA polymerase under the control of a temperature sensitive lambda repressor. The resulting strains were shifted to the nonpermissive temperature and rifampicin was added to turn off transcription from *E.coli* promoters. Subsequent incubation with [35S]-methionine allowed specific expression from the T7 promoter. We observed two protein bands of approximately 14.5 and 10 kd (gel not shown). These bands appeared regardless whether the insert contained either *traA* only, *traA* plus approximately half of the *traL* gene or *traA* plus the complete *traL* gene. We concluded that these two proteins are the product of the *traA* gene.

In F the primary product of the *traA* gene is known to be processed (30). A processing step seemed to be the most logical interpretation for the appearance of a second protein band in our experiment. In order to demonstrate the product precursor relationship a pulse-

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**Figure 6.** Nuclease S1 protection experiment with probes 'C', 'D' and 'E'. RNA from untreated and from cells treated with rifampicin for 30 minutes was included. For the controls tRNA (left lane) and total RNA from plasmid-free *E.coli* K12 5K (right lane) was used. S1 nuclease concentration was 2000 U/ml. The marker is *HpaII* digested pUC119. In lane P the mixture of probes 'C', 'D' and 'E' was separated. Fragments w, x, y and y* refer to probe 'E'. The fragment lengths are the same as in figure 5. y* designates a fragment which is preponderant in this experiment but represents a minor band in figure 5.
chase experiment was done using two different plasmids namely pT7A and pT7AL, which contain either the entire \textit{traA} or the \textit{traA} and the complete \textit{traL} genes, respectively. Figure 3 shows an autoradiogram of electrophoretically separated cell extracts isolated at various time points after the addition of an excess of unlabelled methionine. Whereas the upper band disappears with continuing incubation time, the lower band remains practically constant during the whole incubation period. At the onset of the experiment a weak band of still smaller size could be observed, which is no longer visible upon prolonged incubation. This product may be the smaller cleavage product of the proteolytically processed precursor protein.

\textit{Southern hybridizations}

In order to directly demonstrate the existence of stable mRNA species coded from the region of interest \textit{E.coli} cells carrying Rl-19 were \textit{in vivo} $^{32}$P-labelled. RNA was extracted from these cells after 30 minutes of incubation with rifampicin. The large RNA species were isolated after electrophoresis from polyacrylamide gels and used as probes in a Southern hybridization experiment. As shown in figure 4 no signal was detectable with a deletion containing parts of the \textit{traJ} and \textit{traY} genes as in pEO10. As the DNA fragments became gradually larger in the direction of the \textit{tra} operon, hybridization could be observed. A weak signal appeared in the case of pEO5 which ends just short of the beginning of the \textit{traA} gene. A clear signal was observed with pEO4 which contains

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{S1 nuclease protection experiment with probe 'C' and 'G'. Fragments w', x', y' and y'*', derived from probe 'G', are shorter than the ones of the previous figures but the the protected DNA fragments share the same 3' termini. In track P probe 'G' is shown. All other details are as in figure 6.}
\end{figure}
approximately half of traA. The larger DNA fragments, like pEO3 and pEO2, which contain the complete traA gene or the traA, traL and part of the traE genes, respectively, gave strong signals.

**Sl nuclease protection assays**

In order to determine the stability as well as the exact location of the 5' and 3' ends of the stable mRNA S1-nuclease protection experiments were performed. RNA extracted from E.coli cells harbouring R1-19 was hybridized to various uniformly 32P-labelled DNA probes (Figure 1C). Both rifampicin treated and untreated control cells served as sources for the RNA. DNA probes were obtained by in vitro polymerization off a ssDNA template. Fragments of defined lengths were produced by cleavage with appropriate restriction enzymes. The probes were isolated after electrophoresis from low melting agarose gels and subsequently used for the nuclease S1 protection experiments. After hybridization to RNA and nuclease S1 digestion the remaining nucleic acid mixtures were subjected to electrophoresis on a sequencing gel. Analysis of the autoradiograms allowed the identification of protected DNA segments.

In the first of a series of experiments the stability of the RNA was tested. RNA was extracted at various time points after rifampicin treatment from R1-19 containing cells. These eight RNA samples were allowed to hybridize with two different probes, followed by S1-nuclease treatment. One probe (‘A’, Figure 1C) was protected by the RNA. The protected segments of probe ‘A’ appeared as a series of discrete bands (Figure 5). The DNA fragment designated ‘v’ was present up to 30 min and measures 530 ± 5 nt. Other prominent fragments are those designated ‘w’ (465 ± 3 nt), ‘x’ (425 ± 3 nt), ‘y’ (375–380 ± 2 nt), and ‘z’ (320 ± 2 nt). Fragments ‘t’ and ‘u’, with lengths of 750 ± 10 nt and 630 ± 7 nt, respectively, are due to RNAs of considerable lower stability. Using probe ‘B’ no stable RNA could be detected. Only a short lived transcript was present giving rise to two weak bands of around 500 nt.

Another experiment was designed to locate the 3’- and 5’ termini of the most prominent stable RNAs. From probes ‘C’ and ‘D’ (Figure 1C), differing in their 5’ termini only, protected DNA fragments of the same lengths arose, i.e. 550 ± 5 nt, 515 ± 4 nt, 465 ± 3 nt and 448 ± 3 nt. These bands are the predominant ones both in the rifampicin treated and the untreated samples (Figure 6). Hence, the 3’ terminus of the mRNA must lie within

![Figure 8. Graphical representation of stable RNA molecules as determined by experiments of figures 5 to 8. Wavy lines denote RNA molecules in relation to the genetic and physical maps. The numbers mark the 5' ends and correspond to the numbers of the sequence shown in figure 2.](https://academic.oup.com/nar/article-abstract/17/4/1283/1258033)
the region which is covered by the shorter of these two probes. Probes 'E' and 'F', possessing the same 5'ends as probe 'A' yielded protected fragments of shorter lengths. As can be seen in figure 6 probe 'E' produces fragments 'w', 'x', 'y' and 'y*' of figure 5. Fragment 'y*' measures 360 ±2 nt and is not specifically designated in figure 5 where it represents a minor band. Probe 'F' generates in addition fragment 'v' (data not shown). The distance in length between these fragments is the same as with probes 'C' and 'D'. The shorter fragments obtained with probe 'E' can only be explained by a mRNA whose 3'terminus extends beyond the 5'end of this probe. The reduction in the length of the protected fragment between probes 'C' and 'E' is 90±4 nt. The 5'end of probe 'E' is known from nucleotide sequencing and lies at position 4437. We assume therefore that the 3'terminus of all observed mRNAs lies 90 nt beyond this position, i.e. at nucleotide 4527 ±4. Another important implication of this result is that the heterogeneity of the bands shown in figures 5 and 6 evidently is not due to different 3'termini but rather to various 5'ends of the different mRNA species present in the S1 nuclease assay. The idea that the 3'termini of the mRNAs are constant while the 5'termini vary is supported by another experiment with probe 'G', which is still shorter at its 5'end. The probe was constructed using a specific oligonucleotide, creating a 5'end at position 4206. Protected DNA fragments of 231 nt (fragment w'), 204 and 195 nt (fragment x'), 145, 143 and 140 nt (fragment y') and 132, 130 and 125 nt (fragment y**) were generated (Figure 7). The distance in length to the corresponding fragments originating from probes 'C' and 'D' is 319 ±3 nt; if this length is added to the 5'terminus of probe 'G' one arrives at position 4525 ±3 nt for the common 3'termini of the mRNAs. This value is consistent with the one obtained above. The 5'ends of the mRNAs can be calculated by subtracting the lengths of the various protected DNA fragments from the 5'ends of all probes except 'C' and 'D'. The results are shown in a graphical representation in figure 8.

The lower three bands appearing with probe 'G' are doublets or triplets which are visible

![Figure 9](https://academic.oup.com/nar/article-abstract/17/4/1283/1258033)

**Figure 9.** Influence of S1 nuclease concentration on fragment intensities. Experiment 1 was carried out with 20 μg total RNA and 200 U/ml S1 nuclease, experiment 2 with 10 μg RNA and 1000 U/ml S1 nuclease. In experiment 3 20 μg RNA and 1000 U/ml S1 nuclease were used. All three experiments were carried out with probe 'C'. In a control reaction RNA from R1 carrying cells was tested under the conditions of experiment 3. Marker 1 is the same as in figure 5.
in this lane because of better resolution. The frayed ends are either due to imprecise cutting by S1 nuclease or to slight differences in the mRNA length.

The relative intensity of the bands varies with the concentration of S1 nuclease used in the assay. This is shown in Figure 9. The same DNA/RNA hybrid was treated with 200 or 1000 units of S1 nuclease per ml. More nuclease shifts the intensity of the bands towards smaller sizes. This experiment shows that the relative intensities of the radioactive bands do not necessarily reflect the relative concentration of each of the mRNA species. Figure 9 also shows that RNA extracted from cells harbouring the resistance plasmid R1 does not contain the message for the tra operon. Since the tra genes in R1 are known to be repressed, this result is not surprising.

The traYZ promoter was shown in an earlier investigation (17) to occur at position 3601 of this sequence (corresponding to position 848 in reference 17). The longest transcript in figure 5 measures 750 ± 10 nt, the 5'end of the traYZ mRNA was thus calculated to be at position 3687 ± 10 nt. This position is approximately 90 nt downstream of the traYZ transcription start site determined earlier by primer extension studies. At the moment we cannot decide whether there are still longer transcripts which could not be detected with the method used here or whether the formerly detected promoter is to be categorized as a shadow promoter. Shadow promoters were detected in the homologous region of the F-plasmid (31).

Figure 10. Computer generated (38–40) secondary structure at the 3'terminus of the stable mRNA species. Numbers refer to the sequence in figure 2. The experimentally determined 3'ends are shown by arrows.
DISCUSSION

The *tra* operon of Rl-19 but not that of Rl is transcribed. This finding is not unexpected since Rl-19 is a derepressed mutant of Rl, expressing its conjugation genes constitutively. Quite unexpected was the observation that part of the *tra* operon transcript has an extremely long lifetime which certainly exceeds 30 minutes. Bacterial mRNAs generally show half lifes of 2–3 minutes (1).

The stable regions of the mRNA cover the *traA* gene and two thirds of the downstream *traL* gene, where they apparently possess a common 3’terminus. At their 5’ends the stable mRNAs are heterogeneous reaching in part well into the upstream *traY* gene. Some mRNAs are shorter, however, than the *traA* gene (for details see figure 8).

Since no 5’exoribonucleases are known (6), we conclude from our data that the various 5’termini are generated by an endoribonuclease from a larger precursor molecule. Apparently specific cleavage sites in the primary transcript lead to discrete molecular species which appear as distinct bands in our S1 nuclease experiments. In a progressive way short pieces are removed from the 5’ends of the mRNA and are then rapidly degraded by the known cellular 3’exoribonucleases.

The various mRNAs with differing 5’termini are not equally stable. The longest ones, which contain the entire *traA* and *traY* genes show the shortest apparent half life and are probably successively converted to shorter variants containing only *traA*. The different stabilities of the various mRNA species may be the result of better protection by ribosomes due to higher translational efficiency of the *traA* mRNA. The *traA* cistron is probably translated with a higher efficiency as compared with the upstream lying *traY* gene because the latter is initiated with the rare initiation codon GUG and contains AUA for Ile and AGG for Arg which are absent from the *traA* gene. The appearance of the latter two codons is indicative of genes with a low rate of translation (32,33). Most of the cleavage sites for the *ompA*, *bla* (7) and T4 gene 32 (8) mRNAs were reported to occur in the 5’noncoding regions. This is an indication that these structural genes are also protected by ribosomes. Another example for this mechanism comes from Cole and Nomura, who reported that L11 mRNA decay is stimulated by translational repression (34).

It is known that stem—loop structures can stabilize RNAs in *E.coli* (1). We propose that in the case of the stable *tra* operon messages this mechanism is responsible for the protection of the 3’terminus against exonucleolytic attack. We found an RNA segment which may fold into the structure shown in figure 10. The experimentally determined 3’end of the mRNA coincides with that of the secondary structure. We interpret this correlation to mean that the extensive folding of the mRNA within the *traL* gene acts as a barrier against fast and extensive exoribonucleolytic degradation of the mRNA from the 3’terminus.

The stability of the mRNA is probably related to the amount of protein synthesized and present in the cell. The processed product of *traA* in F-like plasmids is pilin, a protein needed in conjugation proficient cells for the formation of sex-pili which are assembled from repeating units of this protein. A large pilin pool has been found in the inner membrane of F-containing cells but only a small percentage of this pool is utilized for pilus formation (10). Since the *traA* gene is part of the *traYZ* operon containing many other genes which do not need to be expressed at high levels some kind of posttranscriptional positive regulation is needed to meet the specific quantitative requirement for pilin. We propose that on the stable mRNAs repeated cycles of translation occur yielding high levels of the immediate product propilin.

Expression of *traA*, when transcribed from a T7 promoter, resulted in the production
of proteins of the expected size. The smaller one is a processing product of the larger and is very stable. It shows no signs of degradation even after 45 minutes, a property one would expect from an extracellularly occurring structural protein. The spontaneous processing of the immediate \textit{traA} product shows that a host protease or a signal peptidase cleaves the propilin molecule. Obviously, no other plasmid R1-19 coded function is needed for this first processing step.

The \textit{traL} gene could not be detected in the T7 expression system. This could mean that the coding sequence is not intended to be translated at all or else that our experimental system is not sensitive enough to detect low amounts of the protein. It is interesting to note that in other plasmids, like F(35), pED208 (36) and P307 (Schlacher and Hogenauer unpublished) also no \textit{traL} protein could be detected. The possibility has to be considered that the gene acts at the RNA level only, e.g. by providing the secondary structural element discussed previously. However, the high conservation of the ORF among the \textit{traL} genes of various F-like plasmids would argue against this assumption. Furthermore, a gene search by computer using an algorithm of Staden (37) assigned the \textit{traL} ORF a high probability to code for a protein. We see three possibilities why the protein may be translated at such a low rate: lack of a proper RBS which is also absent in pED208, extensive secondary structure of the mRNA as shown in figure 10, or excessive sensitivity towards proteases.

ACKNOWLEDGEMENTS
This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung, grant number P6254. We are grateful to Anton Beyer for the computer analysis.

References