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Characterization of the In Vivo RNA Product of the pOUT Promoter of IS*10*$_R$

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We characterized a single RNA species (RNAout1) which was the major in vivo RNA made from pOUT of IS*10*$_R$. RNAout1 was 70 nucleotides long; its 5′ end corresponded exactly to the in vitro start of pOUT transcription. The concentration of RNAout1 was estimated at 5 to 10 molecules per cell containing the single-copy plasmid NR1. RNA sequences from pOUT of IS*10*$_L$ were detected at a much lower (less than one molecule per cell) steady-state concentration and may be preferentially degraded in vivo. We suggest that the low level of the IS*10*$_L$ transcript led to the inability of IS*10*$_L$ sequences to translationally inhibit Tn*10* transposition.

Transposon Tn*10* encodes bacterial resistance to tetracycline in several naturally occurring bacterial resistance factors including NR1 (R100) (13). The tetracycline resistance gene is flanked by two IS*10* insertion elements. The rightward IS*10* element, IS*10*$_R$ includes three promoters, pIN (the promoter for transposase), pOUT, and pIII (22). Transcription from pOUT extends in the opposite direction from the others, i.e., into sequences external to the Tn*10* element. Genetic studies (23, 24) suggest that RNA species transcribed from pOUT (termed RNAout) negatively control the translation of the transposase by pairing with the mRNA specified by pIN. A function for pIII has not yet been established.

We have previously identified several small RNA species encoded by Tn*10* (7, 21). In the present study, we isolated a small in vivo RNA (RNAout1) which was synthesized from pOUT of IS*10*$_R$. A corresponding product from IS*10*$_L$ was apparently unstable in vivo. The differential metabolic properties of the RNAs from the IS*10* elements may account for the differential ability of the IS*10* elements to repress in vivo Tn*10* transposition in the multicopy inhibition assay.

MATERIALS AND METHODS

Enzymes and chemicals. Enzymes, including restriction endonucleases, *Escherichia coli* DNA polymerase I large fragment, and T4 DNA ligase, were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and New England BioLabs, Inc., Beverly, Mass. [^{32}P]H_3PO_4 was from New England Nuclear Corp., Boston, Mass. DNase I (RNase-free) was from Boehringer-Mannheim Biochemicals, Indianapolis, Ind. S1 nuclease was obtained from Calbiochem-Behring, La Jolla, Calif., or Boehringer-Mannheim. Polynucleotide kinase and calf intestinal alkaline phosphatase were from P-L Biochemicals, Inc., Milwaukee, Wis. [γ-^{32}P]ATP was synthesized by the procedure of Johnson and Walseth (11). [α-^{32}P]dCTP was from Amersham Corp., Arlington Heights, Ill. RNase A, RNase T_1, and RNase T_2 were from Calbiochem-Behring. Cellulose acetate strips and nitrocellulose were purchased from Schleicher & Schuell, Inc., Keene, N.H. DEAE-cellulose paper was obtained from Micro Filtration Systems.

Plasmids and bacterial strains. *Escherichia coli* strains used in this study were N2095 (*thi-1 rpsL9 arg-11 nadB4 lacY1 gel-6 malA1* λ^r *xyl-7 ara-13 mdh-2 mal*$^+$ *rnc*$^+$ [1]) and JA221 (*hsdR hsdM*$^+$ *lacY leuB6* Δ*trpE5 recA1* [from the collection of K. Bertrand]). JM101 (18) was used for growth of M13 derivatives. Plasmid NR1 (FII Cm^r Sm^r Hg^r Tc^r Fs^r) was introduced by conjugation into the *E. coli* strains above. Plasmid pRT61 (12) is the source of the 942-base-pair (bp) fragment containing 395 bp of IS*10*$_R$ and 547 bp of λ DNA (Fig. 1). Plasmids pKP1150 and pKP1131 carry Tn*10* segments in pACYC177 (4); they were constructed by K. Postle and kindly provided to us. In the construction of these plasmids, a *Pst*I fragment containing Tn*10* was cloned into pACYC177 to generate pKP1102 (Tc^r Km^r Ap^r). Digestion of pKP1102 with *Bam*HI and reclosure yielded pKP1150; digestion of pKP1102 with *Eco*RI and reclosure yielded pKP1131. Plasmid pKP1131 (Km^r Tc^s) contains IS*10*$_R$ but not IS*10*$_L$; conversely, pKP1150 (Km^r Tc^s) contains IS*10*$_L$ but not IS*10*$_R$. M13mp7 bacteriophage (18) and replicative-form (RF) DNA were from Bethesda Research Laboratories.

Preparation of single-stranded DNA for hybridization-selection. The 942-bp *Hin*cII fragment of plasmid pRT61 was inserted into M13mp7 RF DNA and the ligated RF DNAs were transfected into *E. coli* JM101 by the method described by Messing (17). The orientation of the insert in M13 recombinants was determined by restriction digestion of the recombinant RFs. Single-stranded DNAs were isolated by polyethylene glycol precipitation and phenol extraction from the supernatants of the culture infected with the respective recombinant phase.

Selection of [^{32}P]RNA complementary to single-stranded DNA probes. The liquid hybridization was done by the method of Bovre et al. (2) with some modification. Total cellular RNAs were labeled with [^{32}P]H_3PO_4 for 1 h at 37°C, isolated, and purified either as described previously (19) or by hot phenol extraction (8). For the tetracycline induction experiment, 10 μg (final concentration) of tetracycline per ml was added 10 min before labeling with [^{32}P]H_3PO_4 (21). Labeled total cellular RNAs were hybridized to 10 to 50 μg of M13 recombinant phage DNA in 30 μl of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate [pH 7.0])–1 mM EDTA–0.2% sodium dodecyl sulfate at 50°C overnight. The RNA-DNA hybrid was collected on a nitrocellulose filter and treated with RNase A and RNase T_1 at room temperature for 1 h. The filter was then incubated with iodoacetate

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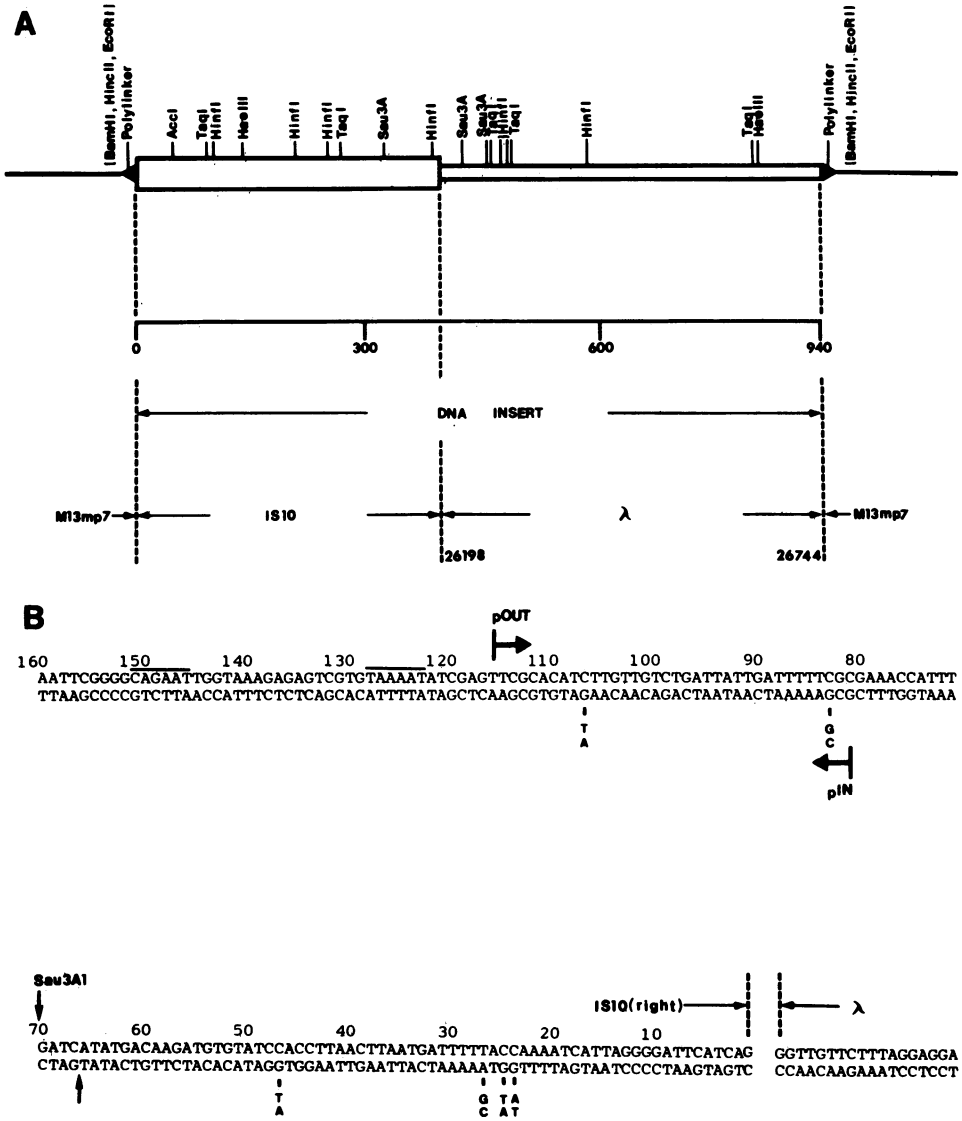

FIG. 1. DNA insert in phages YL10A and YL10B. (A) A 942-bp *Hin*cII fragment derived from pRT61 was cloned into the *Hin*cII site of M13mp7. The resulting recombinant phages were YL10A and YL10B. The sequence of the inserted DNA was deduced by combining the reported sequence of IS*10*$_R$ (9), the predicted λ DNA sequence from the restriction mapping analysis, and the chemical sequencing data of the junction between IS*10*$_R$ and λ DNA (Y. Lee and F. J. Schmidt, unpublished data). The base-pair coordinates on the 5′ to 3′ *1* strand of λ DNA are as indicated previously (6). (B) DNA sequence near the outside of IS*10*$_R$ in the 942-bp *Hin*cII fragment. Differences between IS*10*$_R$ and IS*10*$_L$ are indicated below the sequence. RNA starts from pOUT and pIN; −10 and −35 regions for pOUT, as determined by Simons et al. (22), and the *Sau*3AI site labeled for S1 mapping are marked.

solution (0.15 M sodium iodoacetate, 0.1 M NaOAC, 2× SSC [pH 5.2]) at 48°C for 1 h to inactivate the RNases. RNA was released from the filters at 90°C, precipitated, and applied to a 10% polyacrylamide gel containing 7 M urea.

Analysis of [^{32}P]RNA. RNA bands were cut from the gel, crushed, and extracted with 500 mM ammonium acetate–10 mM magnesium acetate–1 mM EDTA–0.1% (wt/vol) sodium dodecyl sulfate. The gel debris was removed by centrifugation and filtration through glass wool. [^{32}P]RNA was precipitated with ethanol. RNase T_1 fingerprinting analysis was done with the techniques described previously (3). The base compositions of individual RNase T_1 oligonucleotides were analyzed by two-dimensional chromatography on thin-layer cellulose (20).

Preparation of DNA fragments for S1 mapping analysis. DNAs of M13 phages YL10A and YL10B contained the same insert sequences in opposite polarity. When these phage DNAs are hybridized, only the insert sequences are able to form a hybrid; the vector sequences have the same polarity and therefore remain single stranded (17). DNA fragments (250 μg of each single-stranded DNA) were mixed in 500 μl of hybridization solution (0.03 M sodium citrate [pH 7.0], 0.3 M NaCl, 0.2% [wt/vol] sodium dodecyl sulfate, 1 mM EDTA) and incubated for 8 h at 65°C. The solution containing hybridized DNAs was diluted with 2 ml of 0.3 M sodium acetate, and the DNAs were precipitated by the addition of 5 ml of ethanol. The ethanol precipitate was washed two times with 70% ethanol, dried, and used for *Sau*3AI digestion. The digestion was done at 37°C with 50 U of the enzyme in a 500-μl reaction volume. The double-

standed DNA fragments, derived solely from the inserted DNA sequence, were purified by electrophoresis in a 5% polyacrylamide gel. No digestion of the single-stranded vector DNA was observed. The identities of the double-stranded digestion products were confirmed by secondary restriction digestions and chemical DNA sequencing (15). The 5′ ends of the fragment were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (14). For labeling the 3′ ends of the fragment, the following reaction was carried out. DNA (5 to 20 pmol) was incubated with 100 μM dATP–100 μM dGTP–100 μM dTTP–100 μCi of [α-^{32}P]dCTP and 1 U of a large fragment of *E. coli* DNA polymerase I in 25 μl of polymerase buffer (0.01 M Tris hydrochloride [pH 7.5], 0.01 M $MgCl_2$, 0.05 M NaCl, 1 mM dithiothreitol) for 15 min at room temperature. The labeled DNA fragments were digested with a secondary restriction enzyme and purified by gel electrophoresis.

Preparation of cellular RNA. *E. coli* was grown to an optical density at 550 nm of 0.3 in M9 media supplemented with appropriate nutritional requirements and 0.1% Casamino Acids (Difco Laboratories, Detroit, Mich.). The cultures were divided, and tetracycline was added to one portion until a concentration of 10 μg/ml was reached. The cells were grown for 30 min longer at 37°C and were harvested by centrifugation. Total cellular RNA was purified by phenol extraction as previously described (8), except that the isolated RNA was treated with DNase I (10 μg/ml) to remove any DNA contaminants. In some experiments, cultures were pretreated with chloramphenicol (10 μg/ml) for 70 min to increase the plasmid copy number of the cloned IS*10* sequence. This treatment increased the amount but did not otherwise affect RNAout1.

S1 mapping analysis. The labeled DNA probe was hybridized with 300 μg of total cellular RNA in 30 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 0.04 M 1,4-piperazine diethanesulfonic acid [pH 6.4], 1 mM EDTA). The hybridization mixtures were incubated at 70°C for 10 min, cooled slowly to the hybridization temperatures indicated in the figure legends, and allowed to hybridize for 8 h. The hybrids were diluted with 450 μl of S1 buffer (0.06 M sodium acetate [pH 4.5], 0.1 M NaCl, 2 mM $ZnCl_2$) and treated with 15,000 U of S1 nuclease per ml at 37°C. The protected fragments were ethanol precipitated, incubated for 2 to 3 min at 90°C in 10 mM NaOH–1 mM EDTA–80% (vol/vol) formamide–0.1% xylene cyanol FF–0.1% bromphenol blue, and run on a thin sequencing gel alongside chemical sequencing reaction products of the labeled probe (15).

RESULTS

Small RNA species selected with single-stranded DNA probes. To facilitate strand-specific selection of RNA, a 942-bp fragment of plasmid pRT61 containing the outside end of IS*10*$_R$ and hybridizing to tetracycline-induced RNA was cloned into M13mp7 (Fig. 1). The resulting recombinant phages were designated YL10A and YL10B, depending on the orientation of the insert in their RF DNAs. Restriction analysis of YL10A, YL10B, and wild-type M13mp7 RF DNAs with *Hin*fI and *Hae*III (data not shown) indicated that phage YL10A DNA contained a strand of the insert DNA complementary to presumed transcripts from pOUT, whereas phage YL10B DNA contained the other strand, complementary to presumed transcripts from pIN.

E. coli strains containing plasmid NR1 were labeled with [^{32}P]H_3PO_4 (19). [^{32}P]RNA was hybridized with the single-stranded DNA probes, and selected RNAs were separated in a 10% polyacrylamide gel containing 7 M urea. A major RNA band migrating near the xylene cyanol dye marker was selected by phage YL10A DNA but not by phage YL10B DNA or M13mp7 phage DNA (Fig. 2). This RNA therefore could arise by transcription from pOUT. The DNA clones in phages YL10A and YL10B contained λ DNA as well as DNA from IS*10*. Since the cells and plasmid NR1 used here contained no λ DNA, the selected RNAs must have originated from IS*10* sequences. Other selected RNA species migrating faster than the dye marker were observed. Filter hybridization (19) which did not involve RNase treatment yielded a single RNA band migrating near the xylene cyanol dye marker (data not shown), implying that the smaller bands are artifacts of RNase digestion. The faster-migrating RNA species were not examined further.

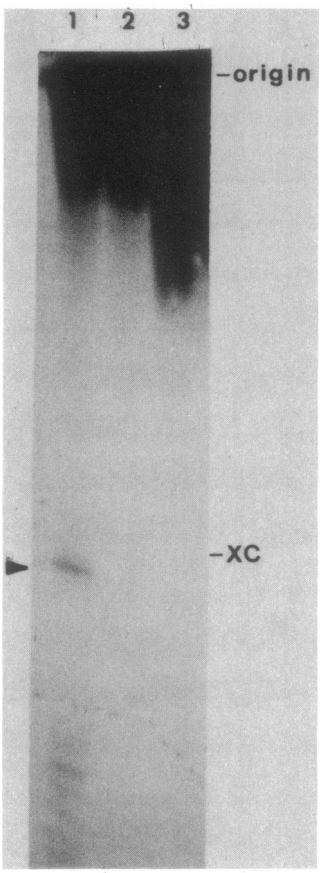

FIG. 2. Hybridization selection of RNA. Equal amounts of [^{32}P]RNA from *E. coli* N2095(NR1) were hybridized to phage DNA. Selected RNA was eluted from the resulting DNA-RNA hybrid, electrophoresed on a 10% polyacrylamide–7 M urea gel, and detected by autoradiography. RNAs were hybridized to YL10A, YL10B, and M13mp7 phage DNAs (lanes 1 through 3, respectively). XC, Xylene cyanol FF which has an electrophoretic mobility equal to that of an extended polynucleotide 70 residues long. Arrow, Position of RNAout1 in the gel.

No RNA smaller than 100 nucleotides was detected by hybridization to YL10B single-stranded DNA. We concluded from these observations that the small RNA product was synthesized from IS*10* with the same polarity as the pOUT transcript. (RNA from pIN would not have been separated from the material near the origin of the gel; its metabolism was not examined in this work.) To characterize the RNA which hybridized to phage YL10A single-stranded

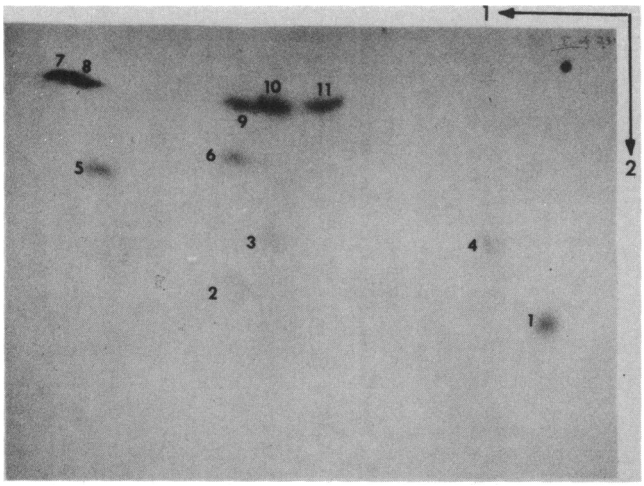

FIG. 3. RNase T_1 fingerprint analysis of RNAout1. RNA was digested with RNase T_1, and the products were separated by electrophoresis at pH 3.5 on cellulose acetate in the first dimension and on DEAE paper in 7% formic acid in the second dimension. Spots 1 to 11 are described in Table 1.

DNA, the hybridization-selection experiment was carried out with 50 mCi of $[^{32}P]H_3PO_4$. The amount of the recovered RNA was 0.0016% of the ^{32}P-labeled total cellular RNA used for hybridization-selection. This value may have been underestimated because the efficiency of the hybridization-selection may not have been 100%. We observed, however, that when nonhybridized RNA from the first hybridization-selection was rehybridized, less than 10% of the RNA was selected, although we cannot exclude the possibility of RNA loss during the manipulation. The selected [^{32}P]RNA (5,000 dpm) was eluted from the gel and subjected to RNase T_1 fingerprint analysis. The movement on two-dimensional electrophoresis (Fig. 3) and the base composition of each RNase T_1 oligonucleotide (Table 1) were compared with RNase T_1 fragments of the predicted transcripts from pOUT of IS*10*$_R$. Since most spots fitted well to a predicted RNase T_1 oligonucleotide catalog of the transcript from pOUT$_R$, we designated the transcript as RNAout1.

The molar yields of several oligonucleotides in the fingerprint deviated from those predicted from the DNA sequence. The discrepancy in molar ratios seemed to result from partial RNase T_1 digestion of RNAout1, probably because of the extensive secondary structure of predicted transcripts from pOUT (9). For example, the sequence G-C-G in the RNA sequence located in a predicted single-stranded region (see below) would have been more susceptible to RNase T_1, resulting in a relatively high molar ratio of C-Gp in the fingerprint.

The RNAout1 termini could not be established exactly by fitting RNase T_1 fingerprint data of the RNA to the gene sequence mainly because of poor resolution of large oligonucleotides on DEAE-cellulose paper. For example, the 5′ terminus of a predicted pOUT transcript was not observed in the fingerprint, probably because of coelectrophoresis with other oligonucleotides. Since RNase T_1 products of RNAout1 contained A-C-A-A-Gp, A-U-Gp, and U-Gp, but not Gp, the 3′ end of RNAout1 was concluded to be between 13 and 52 bp from the outside end of IS*10*$_R$.

Assignment of the 5′ terminus of RNAout1. Nuclease S1 mapping analysis was performed to determine the in vivo 5′ end of RNAout1. A 171-bp ^{32}P-labeled *Sau*3A1-*Hae*III fragment (nucleotides 67 to 241) derived from IS*10*$_R$ (9) was hybridized to total cellular RNA derived from strains containing plasmids which were clones of IS*10*$_R$ or IS*10*$_L$. After nuclease S1 digestion, bands of protected DNA 49 and 50 nucleotides long were observed in the autoradiogram (Fig. 4a). These bands corresponded to the probe protected by RNA species transcribed from the pOUT promoter, which initiates in vitro transcription with pppU at 115 and 116 bp from the outside end of IS*10*$_R$ (22). We concluded that the in vivo transcript from pOUT$_R$ had the same 5′ terminus as the in vitro transcript.

The observed pOUT transcripts were derived from IS*10*$_R$ but not from IS*10*$_L$, even though both IS*10* elements have the same promoter region (Fig. 1B). RNA species capable of protecting the DNA probe were produced by a strain carrying plasmid pKP1131, a clone containing only IS*10*$_R$, but not by a strain carrying plasmid pKP1150, a clone of IS*10*$_L$ (Fig. 4a, lanes 5 and 6). It is possible that RNA from IS*10*$_L$ did not protect the 49- and 50-nucleotide fragments of the probe because of either instability of the DNA-RNA hybrid or S1 nuclease cleavage at mismatches in a hybrid between IS*10*$_L$ RNA and the IS*10*$_R$ DNA probe. In control experiments, however, we did not observe the protected fragment from IS*10*$_L$ when the hybridization mixture was cooled to 4°C and the S1 digestion was performed at 20°C. Neither did we observe a predicted 16-nucleotide DNA fragment that would arise from S1 cleavage at the first mismatch between the IS*10*$_R$ probe and RNA transcribed from the pOUT$_L$ (data not shown). Thus, we concluded that *E. coli* cells did not produce detectable steady-state concentrations of transcripts from pOUT of IS*10*$_L$.

In addition to the 49- and 50-nucleotide-long fragments, we observed protection of the whole DNA probe. These full-length products probably occurred because of RNAs transcribed from external promoters into the IS*10* element. The external promoter was probably the *tet* promoter (10, 25), which is oriented in the same direction as pOUT$_R$. Since the *tet* promoter is strong and is activated by tetracycline, our conclusion that it was the external promoter is supported by the observation that the amount of full-length protected DNA increased when RNA from tetracycline-treated cells was hybridized to the probe (Fig. 4a, lane 2).

3′ end of RNA transcribed from pOUT. A *Sau*3AI-*Hin*fI fragment containing 60 nucleotides from position 7 at the outside end of IS*10*$_R$ was 3′ labeled at the *Sau*3AI site (see Fig. 1). Hybridization of this probe to RNA from *E. coli* containing pKP1131 protected a DNA fragment 24 nucleo-

TABLE 1. Nucleotide compositions of RNase T_1 digestion products

Spot no.[a]	Base composition	Deduced sequence
1	Cp, Gp	C-Gp
2	Up, Gp	U-Gp
3	Ap, Up, Gp	A-U-Gp
4	Ap(3), Cp, Gp	A-C-A-A-Gp
5	Up(2), Gp	U-U-Gp
6	Cp, Up(2–3), Gp	U-C-U-Gp
7	Ap, Cp(1–2), Up(4–5), Gp	A-U-U-U-U-U-C-Gp
8	Ap(2), Up(4–5), Gp	A-U-U-A-U-U-Gp
9	Ap(3), Cp, Up(3–4), Gp	A-U-C-A-U-A-U-Gp
10	Ap(2–3), Cp(2–3), Up(3–4), Gp	A-A-A-C-C-A-U-U-U-Gp
11	Ap(1–2), Cp(3), Up(3), Gp	C-A-C-A-U-C-U-U-Gp

[a] Spots are shown in Fig. 3.

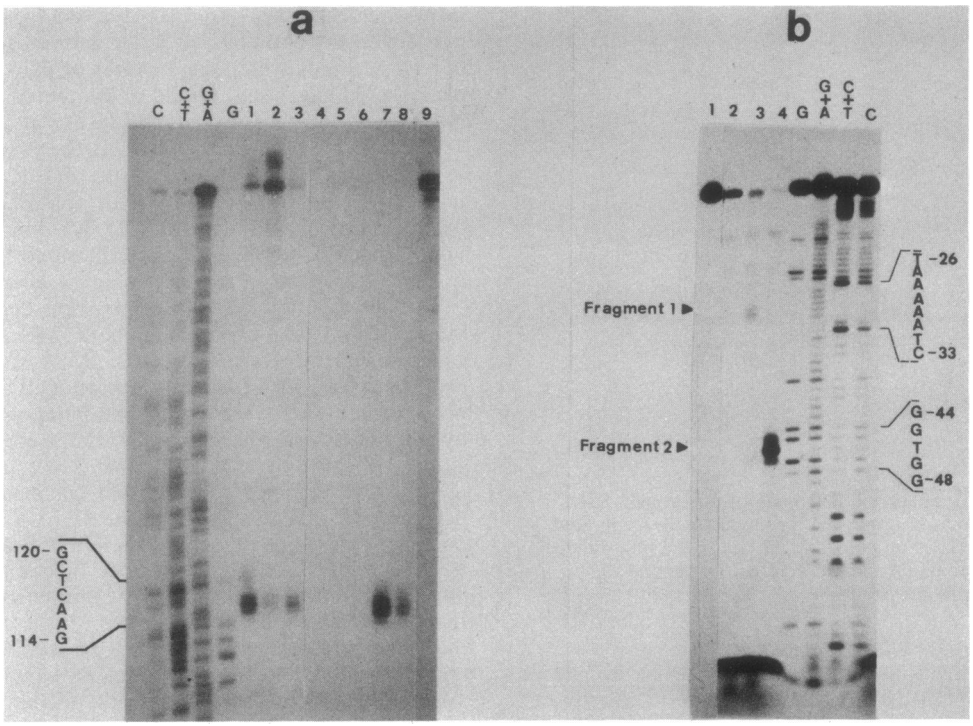

FIG. 4. S1 mapping experiments to identify 5′ and 3′ ends of pOUT transcripts. The nucleotide positions from the outside end of IS*10*$_R$ on the DNA sense strand are indicated on either the right or left side. Chemical sequencing reaction products of the DNA probes are indicated as G, G + A, C + T, and C. (a) The 171-bp *Sau*3A1-*Hae*III fragment, 5′ end-labeled with ^{32}P at the *Sau*3A1 site, was hybridized to total cellular RNA at 37°C. The following RNAs were used for analysis. Lanes 1, RNA from N2095(NR1) treated with chloramphenicol; 2, RNA N2095(NR1) treated with tetracycline; 3, RNA from N2095(NR1); 4, RNA from N2095 containing no plasmid; 5, RNA from JA221(pKP1150) treated with chloramphenicol; 6, RNA from JA221(pKP1150); 7, RNA from JA221(pKP1131) treated with chloramphenicol; 8, RNA from JA221(pKP1131); 9, DNA probe. Since these experiments were done under conditions of RNA excess, the relative diminution of RNAout1 relative to readthrough products protecting the whole probe did not reflect a decrease in the absolute in vivo concentration of RNAout1 (cf. Fig. 5). (b) The 60-bp *Sau*3A1-*Hin*fI fragment 3′ end labeled with ^{32}P at the *Sau*3A1 site was used for S1 reactions. Hybridization conditions and S1 digestion were as described for panel a except that the hybridization mixture was slowly cooled to 4°C. Lane 1 was the DNA probe. Lanes and sources of RNAs used are as follows: 2, JA221; 3, JA221(pKP1150); 4, JA221(pKP1131).

tides long (Fig. 4b). The 3′ end identified by this procedure corresponded to nucleotide 47 of the IS*10*$_R$ sequence. We concluded, therefore, that RNAout1 from IS*10*$_R$ was 70 nucleotides long, extending from nucleotides 116 to 47 of the IS*10* sequence (Fig. 1b). Only a minor proportion of the protected DNA was the full-length product (Fig. 4b, lane 4). Full-length DNA would have been protected by any pOUT transcript >110 nucleotides in length. The low amount of full-length protection thus indicated that RNAout1 was the major pOUT transcript in vivo.

A mapping experiment was carried out to determine if 3′ ends of RNA species from IS*10*$_L$ were detectable. The results contrasted with the 5′-end mapping. In those experiments, no protected DNA band was observed after hybridization of RNA from cells containing IS*10*$_L$, even after prolonged exposure of the autoradiogram. When the 3′ end of RNA from IS*10*$_L$ was analyzed, however, two protected DNA bands were observed in the autoradiogram (Fig. 4b, lane 3). The densities of these bands were much lower than those observed when RNA encoded by pOUT$_R$ was used to protect the DNA probe. This difference in band intensity reflected a lower cellular concentration of RNA derived from IS*10*$_L$. A small amount of protected DNA migrated with the same mobility as the fragment (Fig. 4b, lane 4, fragment 2) protected by RNA from IS*10*$_R$. (The band was visible in the original autoradiogram but not in the photograph.) A second band migrating more slowly in the gel was also observed in the autoradiogram (Fig. 4b, lane 3, fragment 1). The 3′ end of an RNA which could generate fragment 1 corresponds to a site of NusA-dependent termination mapped in vitro by R. W. Simons (personal communication).

It is somewhat anomalous that a 3′ end of RNA from pOUT$_L$ could be observed in an S1 mapping experiment (Fig. 4b, lane 3), whereas the 5′ end of this RNA could not be detected (Fig. 4a, lanes 7 and 8). It is possible that the 5′ end was not observed for technical reasons, because of the low concentration of the leftward RNA in vivo. Alternatively, it is also possible that the 5′ end of RNA from pOUT$_L$ was not detected because the transcript was rapidly degraded from the 5′ end. These two possibilities are not distinguished here; in any case, the total concentration of RNA sequences from pOUT$_L$ could be estimated at less than 5% of the RNA from pOUT$_R$. RNAout1 was about 0.002% of total cellular RNAs from cells containing plasmid NR1, as judged by the yield of RNA after hybridization-selection. Therefore, we estimated that each cell (carrying NR1) had about five copies of RNAout1, assuming that rRNAs constituted 80% of total cellular RNA and that each cell had 5,000 copies of each rRNA under the labeling conditions. The corresponding RNA from IS*10*$_L$ would have been present at a level of less than one copy per cell.

No effect of tetracycline on the level of RNAout1. Previously (21), we identified an RNA from IS*10* that was induced by treatment of the cells with tetracycline. It was, therefore, of

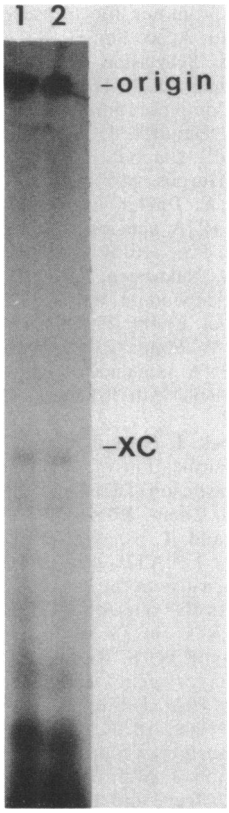

FIG. 5. No effect of tetracycline on RNAout1 synthesis. [^{32}P]RNA was selected against phage YL10A DNA and run on a 10% polyacrylamide–7 M urea gel. Lanes: 1, hybridization-selection with labeled cellular RNA from cells containing plasmid NR1; 2, as in lane 1, except the cells were treated with tetracycline. XC, Xylene cyanol FF dye marker.

interest to examine the effect of tetracycline on the synthesis of RNAout1. We treated cells with 10 μg of tetracycline per ml 10 min before labeling under conditions which induced the IS*10* RNA in minicells. The concentration of RNAout1 was not altered in tetracycline-treated cells (Fig. 4a, lane 2; Fig. 5). Rather, an increase in the amount of RNA protecting the entire probe was observed. Thus, RNAout1 did not seem to be identical with the tetracycline-induced Tn*10* RNA observed in minicells by Schmidt et al. (21).

DISCUSSION

Identification of a small RNA species from pOUT$_R$. RNAout1 was isolated by hybridization to a single-stranded DNA probe. Several lines of evidence indicated that RNAout1 was a small RNA species transcribed from pOUT. First, RNAout1 hybridized only to M13 phage YL10A DNA complementary to transcripts from pOUT, not to M13 phage YL10B DNA complementary to transcripts from pIN (Fig. 2). Second, the RNase T_1 oligonucleotides of RNAout1 were consistent with the deduced RNase T_1 oligonucleotides of a transcript from pOUT (Table 1). In addition, S1 mapping analysis (Fig. 4) showed that RNAout1 was 70 nucleotides long, derived from the transcription initiation site of pOUT. A possible secondary structure of RNAout1 is shown in Fig. 6. It is composed of a long, stable base-paired region ($\Delta G^{0\prime}$ = −16.5 kcal [−69 kJ]/mol) as calculated by the rules of Tinoco et al. [24]) flanked by short single-stranded sequences. The 3′ end of RNAout1 did not correspond to a known transcription termination site. Transcription from pOUT in vitro (22) and in vivo (5) can extend beyond the 3′ terminus of RNAout1. It is therefore likely that the 3′ end of RNAout1 was derived by the processing of longer transcripts from pOUT.

We examined the effect of tetracycline on RNAout1 synthesis since a small RNA (RNA 3t) capable of hybridizing to the outer 400 bp of IS*10* has been shown to be induced in minicells carrying plasmid NR1 after tetracycline exposure (21). We did not observe reproducible tetracycline induction of RNAout1 (Fig. 4a, lane 2, Fig. 5). Nonetheless, the observation that RNAout1 was still present in cells whether they were treated with tetracycline or not treated suggested that RNAout1 was different from RNA 3t. Other RNA species of the same polarity as RNAout1 might be derived by nuclease cleavage of transcripts from the *tet* promoter since the *tet* promoter is activated by tetracycline (10, 25). It is of interest that Simons (personal communication) has observed that inhibition of transposase synthesis can be caused by transcription of RNAout sequences from an upstream *lac* promoter. Tetracycline might therefore reduce Tn*10* transposition frequency by increasing the RNAout concentration; however, no data are yet available to support or disprove this speculation. The concentration of RNAout1 from plasmid NR1 in *rnc*$^-$ strains, deficient in RNase III, was comparable to that in *rnc*$^+$ strains (unpublished data). This implies that RNase III did not play a direct role in the metabolism of RNAout1.

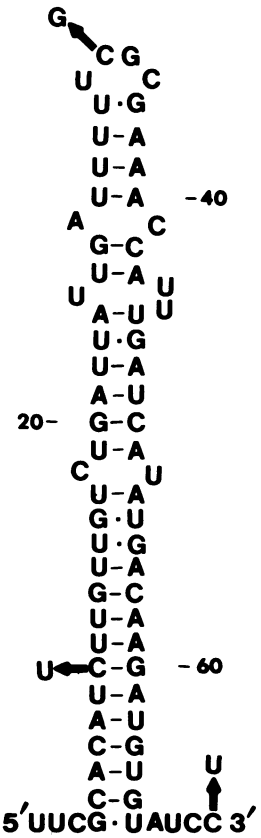

FIG. 6. A predicted stable secondary structure of RNAout1. The base changes in the presumed transcript from pOUT of IS*10*$_L$ are indicated as arrows.

Differential RNA synthesis from $pOUT_R$ and $pOUT_L$. $IS10_L$ does not repress transposition of incoming Tn*10* sequences in the multicopy inhibition assay nor does it translationally repress pIN expression from either $IS10_R$ or $IS10_L$ (Simons, personal communication). In this work, we did not identify an intact RNA transcript from pOUT of $IS10_L$. Why was the steady-state level of this RNA so low (less than one molecule per cell)? Both the hybridization-selection of [^{32}P]RNA and the S1 nuclease mapping experiments measured RNA existing in the steady state (turnover of RNAout1 has not yet been measured). A low (near zero) level of RNAout1 from $IS10_L$, therefore, could have been due to either decreased transcription initiation from $pOUT_L$, premature termination of $RNAout_L$ transcripts, or preferential degradation of the $pOUT_L$ transcript. We favor the latter possibility for several reasons. First, no sequence differences exist between the pOUT promoters of $IS10_L$ and $IS10_R$ (9). Thus, these sequences should function equally well in the initiation of transcription. Consistent with this conclusion are the data of Ciampi et al. (5) which show that Tn*10* can activate adjacent genes in either orientation. This observation also means that premature termination of $pOUT_L$ transcripts does not, by itself, account for the very low concentration of RNA from $IS10_L$. Finally, the sequence of the leftward pOUT transcript contains a base-pair alteration that should destabilize this transcript relative to that of $IS10_R$; the $IS10_L$ RNA would contain a G · U bp in the stem of the helix at nucleotide 10 of the RNA (Fig. 6). This sequence alteration has been predicted to lower the thermal stability of the secondary structure of RNA from $IS10_L$ by about 4.4 kcal (18 kJ)/mol (24).

We suggest that this relative instability is reflected in the lower steady-state level of the RNA from $IS10_L$. As a result, the $IS10_L$ transcripts would be unable to repress translation of transposase mRNA. The equilibrium free energy of an RNA in vitro may not be an accurate predictor of its in vivo metabolic properties. We note, however, that mutant forms of tRNA precursors with similar sequence alterations are aberrantly metabolized and preferentially degraded in *E. coli* (16).

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