Structural analysis of RNA molecules involved in plasmid copy number control

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ABSTRACT

DNA replication of the plasmid ColE1 and its relatives is controlled mainly by a small plasmid-encoded transcript known as RNA1. We have studied the conformation of RNA1 molecules from two related but compatible plasmids, ColE1 and RSF1030, using T₁ ribonuclease, S₁ nuclease, cobra venom nuclease, and diethyl pyrocarbonate modification as probes for secondary structure. Both RNA1 molecules contain three double-stranded stems, three single-stranded loops, and an exposed 5' tail. All loops in both molecules show similar sensitivities to the probes used, as do stems 1 and 3. The region comprising stem 2 of each RNA1 molecule contains the most sequence differences as well as the most structural changes of any region in the two molecules. The structure of the RNA1 molecule encoded by a recessive high copy number mutant of ColE1 was also investigated. Structural alterations involving the first stem and loop of the molecule are proposed to be responsible for the inability of the mutant RNA1 to function in vivo.

INTRODUCTION

ColE1 is a naturally occurring plasmid of E. coli. Normally, each plasmid-containing cell has about 15 copies of ColE1 per chromosome (1). Recent experiments have focused on the mechanism by which the copy number is stably maintained. Genetic and biochemical data have shown that two plasmid encoded RNA molecules are important components of the regulatory machinery (2-7). In vitro, initiation of DNA replication is dependent upon the formation of primer RNA via a two-step process (3). First, a set of primer precursor transcripts is synthesized by RNA polymerase. Each transcript is initiated 555 nucleotides upstream from the origin of replication and terminates at one of several sites past the origin. These precursor molecules are then processed to a mature form by RNaseH (3). Only the processed primer species is capable of efficiently priming DNA replication in vitro. The addition of homologous RNA1 prevents the initiation of DNA replication by blocking the formation of mature primer RNA (4). This inhibition is presumably a consequence of an RNA1-primer interaction which

prevents the processing of precursor primer molecules to mature species by RNaseH (5). Thus, RNAI negatively controls plasmid copy number by regulating the amount of primer available to the replicating machinery. In addition, RNAI is a key molecular component in plasmid incompatibility (4, 5). In vitro, RNAI molecules from compatible plasmids are capable only of inhibiting primer formation of the plasmid from which they are derived. That is, RNAI species from incompatible plasmids cross-react with their respective primer precursors; those from compatible plasmids do not.

Understanding how RNAI interacts with the primer precursor and modulates its processing requires a detailed knowledge of the conformation of these molecules both before and after they interact. A preliminary analysis of ColEI RNAI secondary structure was carried out by Morita and Oka (8). They performed partial T1 digestions on uniformly labeled RNAI, characterized the resulting oligonucleotides and proposed a secondary structure model for the molecule. Using this model as a starting point we undertook a more detailed analysis of the structure of ColEI RNAI. In addition, the RNAI molecule from the plasmid RSF1030 was studied. RSF1030 is compatible with ColEI in vivo; however, the RNAI genes of these plasmids are 75% homologous in sequence. By comparing the structures of these two largely homologous RNAI species we hoped to localize regions in the molecule that were responsible for their functional specificity. Finally, this paper provides a data base to study the structure of mutant RNAI species which should permit detailed correlation of structure and function for this unique regulatory molecule. As an initial step in this process we analyzed the structure of the RNAI molecule encoded by a recessive, high copy number mutant of ColEI. We show that this mutant produces a larger RNAI species in vitro as a consequence of faulty transcription termination. Our analysis demonstrates that the mutant molecule has structural alterations which could account for its inability to function in vivo.

MATERIALS AND METHODS

Plasmids

Plasmid DNAs used as templates for in vitro transcription were isolated as described (9, 10). Either ColEI or the 4.2 kb ColEI derivative pNOP42 (2) was used as the source of ColEI RNAI. For preparation of RSF1030 RNAI the plasmid JN87 was used (11). This plasmid was kindly provided by Dr. June Grindley. pOM5, a recessive high copy number mutant of ColEI, was used as template for pOM5 RNAI production. All templates used were supercoiled.

Preparation of 5' End Labeled RNA1

Plasmid DNA was transcribed in vitro under the following conditions (30 μ l reactions): 50 mM Tris-HCl pH 7.5, 100mM KCl, 10mM MgCl₂, 1mM DTT, 200 μ M each GTP, CTP, and UTP, 40 μ M ATP, 400-800 μ Ci γ -³²P ATP (ICN, carrier free), 300 units/ml E. coli RNA polymerase (New England Biolabs), 60 μ g/ml DNA. After incubation at 30° for 1 hr the reaction was stopped by the addition of 30 μ l sample buffer (80% formamide, 16mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue), heated to 100° for 1 minute, chilled on ice, then loaded on a 4% acrylamide 7M urea gel. Following electrophoresis, the RNA1 band was located by autoradiography, excised, and eluted with tRNA carrier. After ethanol precipitation the RNA was dissolved in water and stored at -20°. For analytical purposes, transcripts were labeled with α -³²P UTP using nucleotide concentrations of 200 μ M each ATP, GTP, and CTP and 40 μ M UTP. In addition, DNA template and RNA polymerase concentrations were five- and ten-fold lower, respectively, and the period of incubation was 30 minutes.

Digestion of RNA for Structural Analysis

T₁ Partial Digestions. RNA1 was incubated in 50mM Tris-HCl pH 7.6, 10mM MgCl₂ for 5 minutes at 37°. T₁ ribonuclease (Sankyo) was added (0.005 units/ μ g RNA) and incubation continued for 1 minute. The reaction was stopped by the addition of an equal volume of sample buffer and frozen at -70°.

S₁ Partial Digestions. Labeled RNA1 was incubated in 25mM sodium acetate pH 4.8, 100mM NaCl, 10mM MgCl₂ containing 1 unit of S₁/ μ g RNA. The reaction was incubated at 37° and at various times aliquots were withdrawn and processed as described above.

Cobra Venom Nuclease Partial Digestion. Digestions were carried out in 10 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 200 mM NaCl at 37° (12). Following digestion the reactions were processed as above. The enzyme used for these studies was generously provided by Dr. John Vournakis (Syracuse University).

Chemical Modification of Adenine Residues with Diethyl Pyrocarbonate. Reactions were carried out as described by Peattie and Gilbert (13). The following conditions were used during the modification reaction: native, 50mM sodium cacodylate pH 7.0, 10mM MgCl₂, 37°; semidenaturing, 50mM sodium cacodylate pH 7.0, 1mM EDTA, 37°; denaturing, identical to semidenaturing except that incubation was at 90°. Diethyl pyrocarbonate (Sigma) was redistilled before use.

Digestions to Produce Markers. To serve as electrophoretic markers, RNA1 was digested with T₁ under denaturing conditions as previously described

(14). Alkaline ladders were generated by incubation of RNA1 in 50mM NaOH, 1mM EDTA at 100° for 30-60 seconds. Reactions were stopped by the addition of sample buffer.

Gel Electrophoresis and Autoradiography

Reactions were run on 12% or 20% acrylamide gels containing 8M urea. 20% gels were run at 1000V for 5 hours. For 12% gels, electrophoresis was at 800V for 12 hours (40 cm gels) or 1500V for approximately 20 hours (1 meter gels). All samples (except alkaline ladders) were heated to 100° for 1-2 minutes then chilled on ice prior to loading. Autoradiography was at -70° with Dupont intensifying screens.

3' End Determination

Sequences at the 3' ends of RSF1030 and pOM5 RNA1 molecules were determined by digesting α -³²P-UTP-labeled RNA to completion with T₁ ribonuclease and separating the oligonucleotides by two dimensional homochromatography (15). Spots were excised from the second dimension plates and redigested with ribonuclease T₂. The resulting mononucleotides were characterized by chromatography on cellulose plates (15). Nearest neighbor frequencies were used to determine the 3' ends. The determinations are accurate to ± 1 nucleotide.

Computer Generated Structural Models

The program of Zucker and Stiegler (16) was used to generate RNA1 secondary structure models.

RESULTS

Structural Analysis Using Ribonuclease T₁

When the conformation of ColE1 RNA1 was probed with ribonuclease T₁ under native conditions, the results shown in Figure 1 were obtained. G residues at positions 4, 24, 55, 56, 88, and 89 were highly susceptible to digestion by the enzyme. These residues can be assigned to the 5' tail region (G4) or the loops of RNA1 as follows; G24, loop 1, G55, G56, loop 2, and G88, G89, loop 3. (See Figure 4 for summary of cleavages.)

Interestingly, other G residues which are also present in loops and are located near these highly susceptible G residues are not themselves cleaved very efficiently. For example, G residues at positions 27, 59, and 92 are cleaved at a much lower frequency than the highly susceptible G residues. These nucleotides are all on the 3' side of their respective loops. These results imply that not all of the bases in the loops are equally exposed to the enzyme. Further evidence to support this conclusion was obtained from S₁

Figure 1. T_1 partial digestion of ColE1 RNA. Left: 20% gel of digestion products. Lane 1, T_1 partial digestion under nondenaturing conditions; lane 2, T_1 partial digestion under sequencing conditions, lane 3, alkaline hydrolysis; lane 4, minus enzyme control. Right: 12% gel of digestion products. Lane 1, alkaline hydrolysis; lane 2, T_1 partial digestion under sequencing conditions; lane 3, T_1 partial digestion under nondenaturing conditions; lane 4, minus enzyme control. Numbers indicate the position of G residues relative to the 5' end of the molecule.

digestion data (see below). The remainder of the G residues in the molecule are either weakly cleaved (G10, G11) or not cleaved at all. This is consistent with their location in base-paired regions of the molecule. In general, our results agree with those reported previously (8) by Morita and

Oka. They reported that G residues 56, 59, and 89 were the most susceptible to cleavage by T_1 . However, they did not observe strong cleavages at residues 4 or 24. The basis for these differences is not clear.

When RSF1030 RNAI was probed with T_1 , results were obtained that were similar to those for ColEI RNAI (results not shown). Figure 4 summarizes the results. G residues located in the 5' tail (G4) or in the loop regions of the molecule (G54, 55, 87, and 88) were most readily cleaved. As with ColEI, the G residues located on the 3' sides of loops 2 and 3 (nucleotides 58 and 91 in RSF1030) were protected from cleavage despite the fact that nearby bases were cleaved strongly.

The T_1 partial digestion results support a model in which both RNAI molecules contain three single-stranded loops and an exposed 5' tail. In addition, G residues located on the 3' sides of the loops are less accessible to cleavage by T_1 , perhaps due to limitations imposed by the tertiary structure of the molecule.

Structural Analysis Using S_1 Nuclease

To probe the single-stranded regions of ColEI RNAI, partial S_1 digestions were performed (Figure 2). Two points are worth noting. First, the major cleavages occur either at the 5' end of the molecule (residues 1, 2, and 3) or in the middle of the loops (residues 23, 24, 25, 55, 56, 57, 87, 88, and 89). At later times, cleavage can also be seen on the 5' side of loop one (nucleotides 20, 21, 22). Cleavage is never observed at the two nucleotides which constitute the 3' portion of a loop even if digestion is carried out for 30 minutes (data not shown). These data are consistent with those obtained with T_1 and suggest that nucleotides on the 3' side of the three loops are inaccessible to these probes. Second, cleavage can be seen in the middle of stem two (nucleotides 44, 45, 46, 47, 48, 49). This region of the molecule contains a run of five adenine residues, three of which could potentially participate in A-U basepairs (see Figure 4). The fact that this region of the molecule is slightly susceptible to S_1 is not surprising due to its high A-U content. However, the enzyme does not cleave on the 3' side of stem two at the nucleotides which oppose those which are cut (nucleotides 63, 64, 65, 66, 67). As can be seen in Figure 2, this portion of the molecule is completely S_1 resistant. One possible explanation for this observation is that the size of S_1 (36k daltons) prevents it from reaching all areas of the molecule that are single-stranded. For example, if the 3' side of stem two was oriented toward the interior of the molecule, S_1 might be sterically prevented from cutting that region. Alternatively, the 3' side of stem two

Figure 2. S_1 partial digestion of ColE1 RNA1. Left: 20% gel of digestion products. Lane 1, S_1 partial digestion for 5 minutes; lane 2, S_1 partial digestion for 1 minute; lane 3, alkaline hydrolysis; lane 4, T_1 sequencing digestion; lane 5, minus enzyme control. Right: 12% gel of digestion products. Lane 1, minus enzyme control; lane 2, S_1 partial digestion for 5 minutes; lane 3, S_1 partial digestion for 1 minute; lane 4, alkaline hydrolysis; lane 5, T_1 sequencing digestion. Numbers indicate the position of G residues relative to the 5' end of the molecule.

may not have single-stranded character due to a tertiary interaction. The inaccessibility of this region of RNA1 is further substantiated by the inability of T_1 to cleave at position G65 and by the lack of reactivity of

residue A66 with diethyl pyrocarbonate (see below).

When the RNA1 molecule from RSF1030 was probed with S₁, essentially the same pattern of cleavages was obtained as for ColE1 RNA1 (results not shown). One minor difference seen in RSF1030 RNA1 was that the susceptibility of loop one seemed to be less than that observed in ColE1 RNA1. The fact that the two RNA1 molecules contain different sequences in this region could be responsible for the different cleavage pattern. However, the preference toward the 5' side of the loops was maintained, qualitatively giving the same pattern seen with ColE1 RNA1. Another slight difference in S1 sensitivity between the two molecules was observed in stem two. Whereas ColE1 RNA1 was cleaved only at late times in this region (Figure 2), RSF1030 RNA1 was cleaved at earlier time points. Experiments using cobra venom nuclease and diethyl procarbonate to probe secondary structure also suggest that the two molecules differ slightly in the conformation of stem two (see below and Figure 4).

Structural Analysis Using Cobra Venom Nuclease (CVN)

The data obtained from the T₁ and S₁ digestions gave some indication as to which regions of the RNA1 molecule contained single-stranded character. In order to complement these data, CVN was used to probe the conformation of RNA1. This enzyme cleaves nucleotides which are either part of double-stranded RNA structures or are involved in tertiary interactions (17). The results are shown in Figure 3 for ColE1 RNA1 and summarized in Figure 4. The strongest cleavages in stem one are at nucleotides U14, U15, A33, and U36; the strongest in stem two are at A48 and A49; the strongest in stem three is at A82. At slightly later times of digestion, each of these strong cleavages is flanked by weaker ones. Note that not all nucleotides involved in Watson-Crick base-pairs are cleaved by the enzyme and that CVN does not always produce symmetrical cuts on both sides of a given stem. Similar observations have been made with other RNA species and presumably result from steric constraints imposed by the tertiary structure of the molecule (12, 17, 18). The majority of CVN cleavages occur at nucleotides involved in base-pairs with one exception. U14 is cleaved strongly, but is unlikely to form a base-pair with U35 on the opposite side of stem one. Perhaps the fact that these two nucleotides are flanked by canonical base-pairs imparts a base-paired-like structure on U14 and U35. Alternatively, since CVN has been shown to cleave nucleotides that are involved in tertiary interactions (17), these bases could be participating in a long-range interaction. CVN also produces minor cuts in some single-stranded regions of the molecule, perhaps

indicating involvement of these nucleotides in tertiary interactions. Two cleavages (A3 and G27) are noteworthy. The observation that neither T₁ nor S₁ cleaves RNAl at position G27 is possibly explained by its involvement in a tertiary interaction. In tRNA molecules, nucleotides involved in tertiary interactions are generally resistant to cleavage by single-strand-specific enzymes (14, 17). The fact that CVN can cleave at position G27 whereas T₁ and S₁ cannot would seem to rule out the possibility that this nucleotide is not cleaved by single-strand-specific nucleases for purely steric reasons since CVN is approximately the same size as S₁ and larger than T₁. The cleavage at G27 is important because the analogous region of the RSF1030 RNAl molecule is also cleaved by CVN (see below). Likewise, cleavage at nucleotide A3 is common between the two RNAl molecules. These results suggest that the tertiary configurations of both RNAl molecules are similar.

When RSF1030 RNAl was probed with CVN (Figure 3), some similarities and differences were seen relative to Cole1. As seen with Cole1, stem one is cleaved most frequently and stems two and three are cut less often. However, two of the major cuts in stem one of Cole1 RNAl occur at presumably non-base-paired nucleotides (U14 and U35). Analogous cleavages take place in RSF1030 RNAl at nucleotides A14 and U34 (Figure 4). These results suggest that the general orientation of stem one is the same in both molecules despite slight differences in sequence.

The cleavage pattern of stem two shows the most differences between the two molecules. Cole1 RNAl has two major (A48, A49) and several minor cleavages (G50, A51, U61, U63), all of which are located at the top of the stem in base-paired regions. RSF1030 has only one strong cleavage site in this area which occurs at a non-base-paired nucleotide (A48) and several minor cleavages in the bottom half of the stem (U44, A67, C68). Because there are a number of sequence differences between the two RNAl molecules in the region of stem two, this result is not surprising. Whether or not these observed structural differences are critical to RNAl function is not clear.

With respect to potential tertiary interactions, RSF1030 RNAl shows cleavages at A3 and C25/C26 which correspond to those seen in Cole1 RNAl at nucleotides A3 and G27. All of these cleavages are weak but reproducible. RSF1030 RNAl has a potential tertiary interaction detected at U70 that does not appear in Cole1 RNAl. The appearance of this new tertiary interaction could be a result of the altered structure of stem two in RSF1030 RNAl relative to Cole1 RNAl.

Structural Analysis Using Diethyl Pyrocarbonate (DEP)

Peattie and Gilbert (13) have shown that RNA conformation can be probed using various base-specific chemicals. By performing DEP modification under native, semidenaturing, and denaturing conditions, it is possible to observe the reactivities of each A residue as the structure of the RNA molecule is progressively disrupted. Studies on yeast tRNA^{Phe} have shown that this approach allows the determination of residues involved in secondary or tertiary interactions (13). The results for ColEI RNAI are summarized in Table 1. When the molecule is modified under native conditions, only a few of the A residues react, namely those at the 5' end (nucleotides 1, 3, and 6) and those in the loops (nucleotides 28, 58, and 91). This indicates that these residues are not involved in either secondary or tertiary interactions. The fact that S₁ and T₁ experiments failed to show the single-stranded nature of the 3' portion of the loops but DEP modification does is likely due to the fact that DEP is a small molecule and has fewer steric restrictions. When the RNAI molecule is modified under semidenaturing conditions (i.e., tertiary interactions disrupted), no new cleavages appear, indicating that none of the A residues participate in tertiary interactions. Modification under totally denaturing conditions detects all A residues in the molecule as expected. With the exception of A3 and A66 (see below), each A residue reacted in a manner consistent with the T₁, S₁, and CVN experiments. It is unclear why nucleotide A3 appears to be involved in a tertiary interaction as detected by CVN but not by DEP modification.

The results for RSF1030 RNAI (Table 1) are similar to those for ColEI RNAI in that A residues at the 5' end (1, 3, and 5) and those in the loops (27, 57, and 90) are all reactive under native conditions. In addition, A73 is reactive, indicating that this nucleotide is not base-paired as is the corresponding residue in ColEI RNAI (A74). This fact, taken together with

Figure 3. Cobra venom nuclease digestion of ColEI and RSF1030 RNAI molecules. A: 20% gel of ColEI RNAI digestion products. Lane 1, CVN digestion for 15 minutes; lane 2, CVN digestion for 5 minutes; lane 3, T₁ sequencing digestion; lane 4, alkaline hydrolysis. B: 12% gel of ColEI RNAI digestion products. Lane 1, minus enzyme control; lane 2, alkaline hydrolysis; lane 3, T₁ sequencing digestion; lane 4 CVN digestion for 5 minutes; lane 5, CVN digestion for 15 minutes. The CVN cleavage pattern in the vicinity of nucleotide 70 has been confirmed from other gels. C: 12% gel of RSF1030 RNAI digested with CVN. Lane 1, T₁ sequencing digestion; lane 2, alkaline hydrolysis; lane 3, CVN digestion for 5 minutes; lane 4, CVN digestion for 15 minutes; lane 5, minus enzyme control. Numbers indicate the position of G residues relative to the 5' end of the molecule.

Figure 4. Summary of cleavage patterns for ColE1 and RSF1030 RNAI molecules. The size of the arrow indicates the extent of cleavage. For clarity, only the major cleavage sites are shown.

the observation that G72 is slightly sensitive to T₁ digestion (see Figure 4), suggests that RSF1030 RNAI contains a short single-stranded region between stems two and three which is not present in ColE1 RNAI. Another important difference between these RNAI species is seen in stem 2. Adenine residues at positions 46, 47, and 48 of RSF1030 RNAI are all clearly reactive under semidenaturing conditions, indicating their involvement in tertiary interactions. This is in good agreement with the cleavage of A48 by CVN as noted earlier and supports the conclusion that the structure of stem two is different in the two RNAI species.

Residue A66 in ColE1 RNAI and its counterpart A62 in RSF1030 RNAI showed an unexpected lack of reactivity to DEP. It was expected that these nucleotides would react under native conditions but neither showed any reactivity except under totally denaturing conditions. This result indicates that this region of stem two appears to be completely double-stranded even when probed with a small molecule, such as DEP, and is consistent with the lack of cleavage in this region by S1 and T1.

Structural Analysis of RNAI from a Recessive Copy Number Mutant

Spontaneous high copy number plasmid mutants of ColE1 have been isolated by selecting for elevated antibiotic resistance (19). One such mutant, pOM5,

Table 1. Summary of DEP modification results for ColE1 and RSF1030 RNA1 molecules.

A Residues Modified by DEP Under:	Source of RNA1	
	ColE1	RSF1030
Native Conditions	1, 3, 6, 28, 58, 91	1, 3, 5, 27, 57, 73, 90
Semidenaturing Conditions	1, 3, 6, 28, 58, 91	1, 3, 5, 27, 46, 47, 48, 57, 73, 90

contains a single nucleotide change within the third stem of RNA1 (Figure 4). The mutation, a C → U transition at position 93 of RNA1, destabilizes the third stem and allows readthrough transcription past the normal RNA1 terminator both *in vitro* (Figure 5, lane 3) and *in vivo* (unpublished results). These results are similar to those observed with another high copy number mutant, pOP1Δ6, which changes position 98 from a G to a U in RNA1 (Figure 5, lane 2). The pOM5 readthrough RNA1 species labels with (γ-³²P) ATP suggesting that it is initiated at the normal RNA1 start site. This fact was directly established by sequencing and fingerprinting experiments (data not shown) which demonstrated that this molecule has an additional 54 nucleotides at its 3' terminus compared to wild type RNA1.

pOM5 behaves as a recessive copy number mutant *in vivo*; i.e., it is displaced by a wild-type ColE1 plasmid (results not shown). The elevated copy number of pOM5 might be due to the fact that the readthrough transcript is nonfunctional. To understand the basis for the recessive phenotype, we investigated the structure of the isolated readthrough molecule of pOM5. Computer-generated secondary structure models indicated that sequences downstream from the normal RNA1 termination site were capable of forming alternative stem and loop structures with the initial 40 nucleotides of RNA1. These alternative structures would preempt the formation of the usual stem and loop 1 region of RNA1 (Figure 6). When the conformation of this RNA was probed enzymatically (T₁ and CVN), the data were consistent with the structure predicted by the computer (data not shown). Figure 6 summarizes the results. Notice that G24, which is highly exposed to T₁ digestion in the wild type molecule, is only moderately susceptible in the readthrough

Figure 5. *In vitro* transcription of plasmid DNA. RNA was synthesized as outlined in Materials and Methods and separated on a 4% polyacrylamide gel containing 7 M urea. The following templates were used: lane 1, NOP42; lane 2, pOP1Δ6; lane 3, pOM5. The position of the readthrough transcript (RTH) is marked.

transcript. In addition, G30, G34, and G44 all show increased sensitivity to T_1 relative to the wild type molecule. G residues located 5' to G24 and 3' to G44 react identically in both molecules indicating that the structural alteration is confined to a small portion of the readthrough RNA. Analysis with cobra venom nuclease further establishes the fact that structural differences exist between the two molecules in a limited region. The two strongest cleavages in stem 1 of wild type RNA1 occur at nucleotides A33 and U36 (Figure 4). These cleavages are greatly reduced in the readthrough transcript (Figure 6). Instead, strong cleavages occur at C15 and U16, plus weaker ones at U14, U21, C22, U23, C25, and U26. Most of these weaker

Figure 6. Summary of cleavage patterns for the pOM5 readthrough transcript. Cleavages located toward the 3' end of the molecule which are marked with an asterisk are accurate to ± 1 nucleotide.

cleavage sites are not seen in wild type RNA1 but their appearance in the readthrough transcript is in agreement with the computer generated model. As is the case with T₁, the major difference between the CVN cleavage patterns of the readthrough and wild type molecules is confined to the region comprising stem and loop 1 (of the wild type molecule). The slight alteration in CVN cleavage preference seen in stem 3 of the readthrough transcript (nucleotides 79-82) is probably due to small changes in the stem caused by the mutation at position 93. However, this alteration is minor compared to those seen in the region of stem and loop 1. With the exception of the strong susceptibility of G43 and G44 to T₁ cleavage, the enzymatic data agree well with the computer model. The data for G residues 43 and 44 can be accommodated if one allows for melting or instability of the bottom portion of stem 2.

DISCUSSION

The results reported here permit establishment of a detailed secondary structure model for the small replication control RNAs from the ColE1 and RSF1030 plasmids. Both RNA molecules have similar overall structures being composed of three double-stranded stems and three single-stranded loops with an exposed 5' tail. In addition, RSF1030 RNA1 has a short single-stranded region between stems two and three which is absent in ColE1 RNA1 (see Figure 4). All three loops in both molecules showed similar cleavage patterns, with nucleotides in the central portion of the loops being the most strongly cleaved by T₁ and S₁. Neither enzyme cleaved nucleotides located toward the 3' side of a loop. Cleavage with the double-strand-specific enzyme CVN revealed similarities in structure between the two molecules in the regions comprising stems one and three. However, CVN gave different cleavage patterns for the RNA1 molecules in the area comprising stem two. This area contains numerous sequence differences between the two molecules (see Figure 4).

Probing with CVN as well as DEP allowed detection of several potential tertiary interactions within the two molecules. Nucleotides A3 and G27 in ColE1 RNA1 and A3, C25, C26, A48 and U70 in RSF1030 RNA1 appeared to be involved in tertiary bonds as defined by CVN cleavage at those sites. DEP modification revealed potential tertiary interactions at residues A46, A47 and A48 in RSF1030 RNA1. Thus, some but not all tertiary bonds are common between the two molecules.

The results obtained on the readthrough transcript from the recessive

copy number mutant pOM5 (Figure 6) demonstrate that this molecule has different secondary structural features than wild type RNAl. As Figure 6 shows, sequences normally present in loop 1 of wild type RNAl are buried in a stem region in the readthrough transcript. Because point mutations in the loops of RNAl have been shown to decrease the activity of this molecule (5), it seems likely that burying those same sequences in stems would have the same effect.

The existence of related but compatible plasmids, such as ColE1 and RSF1030 permits a comparison of related RNAl structures that no longer interact with identical specificity. Both molecules share a high degree of structural similarity. Loops 2 and 3 are identical in sequence, and have virtually identical responses to the conformational probes used here. The main differences between the two molecules lie in the sequence of loop 1 where 4 of 7 residues differ, and in the structure and conformation of stem 2. Thus both sequence and structural differences could play a role in determining the specificity exhibited by the two molecules.

It has been suggested that the loops of the ColE1 RNAl molecule are responsible for the activity and specificity of its interaction with the primer precursor RNA (5, 7). Our results show that the middle region of all three loops is exposed to the conformational probes used here and therefore could be available to participate in an intermolecular RNA-RNA interaction. In addition, nucleotides at the 5' end of RNAl are exposed and could also be involved in the interaction (see below).

Determination of the essential nucleotides required for RNAl function requires separation of mutational effects on the primer from those on RNAl. This can be accomplished by cloning the ColE1 RNAl gene in a compatible plasmid and introducing mutations that alter its ability to display incompatibility towards a co-resident ColE1 plasmid. These experiments have recently been carried out (20). A series of RNAl mutants were isolated with a variety of sequence alterations at the 5'-terminus. Three of these mutants are of interest because they have sequence alterations entirely confined to the 5'-tail region of RNAl, a region where mutations had not been previously detected. Work is currently in progress on the secondary structural features of these mutant RNAl species.

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