Structural analysis of an RNA molecule involved in replication control of plasmid R1

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ABSTRACT

The replication control circuit of the FII plasmids R1, R100 and R6-5 involves a small untranslated RNA molecule of about 93 nucleotides. This RNA, designated CopA RNA in plasmid R1, acts *in vivo* as a post-transcriptional inhibitor of the expression of the RepA protein whose synthesis is required for each round of plasmid replication.

We have studied the structure of CopA RNA in solution by probing with single-strand- as well as double-strand-specific nucleases. Our data show that CopA RNA has two stem-loop structures connected by a long spacer region and a single-stranded 3'-tail.

Enzyme reactions were performed under buffer conditions where we find strong and specific binding of CopA RNA to its target RNA (to be published elsewhere), and the patterns were compared to digests in two other buffer systems. All partial cleavages supported the proposed secondary structure.

We also analyzed the stem-loop regions of CopA RNAs from two R1 copy number mutants. The data show that both RNAs have altered loop structures. The implications of these findings are discussed.

INTRODUCTION

Replication control in plasmids R1, R100 and R6-5 involves a negative control loop (1). The synthesis of a positively required component, the RepA protein, is regulated by the *copA* gene, the product of which is a small untranslated RNA that has been identified *in vitro* and *in vivo* (2,3). Mutations leading to altered copy numbers and the finding that mutations in the *copA* sequence create new incompatibility properties suggest that both the target (CopT) and CopA RNA are coded for by the same DNA sequence (fig. 1). The *copA/copT* region is contained within a 262 bp *Sau3A* fragment located inbetween the RepA promoter and the RepA coding sequence. The direction of CopA transcription is opposite to that of the RepA mRNA such that CopA RNA is completely complementary to the leader region of RepA mRNA.

In plasmid R1, replication control is accomplished via a post-tran-

scriptional control mechanism (4). Most likely, an interaction between the inhibitor CopA RNA and its target (RepA mRNA) in the region of complementarity decreases or prevents translation of the RepA message. There are so far no data supporting any model as to how a hypothetical interaction between two RNAs can affect translational activity.

A precedence for RNA-RNA interactions in plasmid replication control has been studied in detail for ColE1 (5,6), where complex formation between a small countertranscript (RNA I) and the preprimer for DNA replication results in inhibition of primer maturation at a distal site (origin of replication). In ColE1, a primary interaction between RNAs I and II occurs at the single-stranded loops. The loop II in CopA RNA is presumably involved in a recognition reaction in a similar way, suggested by the location of many copy number mutations in or near that loop (7).

We have attempted to study the interaction between CopA RNA and its target to establish the mechanism by which RepA protein synthesis is inhibited. Using in vitro transcribed CopA RNA and a CopT RNA fragment we observed strong and specific complex formation (to be published elsewhere). In order to understand the effects of such an interaction, for example on the secondary structure of the mRNA at sites important for ribosome binding, we need to learn more about the structures of the components involved.

Here we report on the secondary structure of in vitro transcribed CopA RNA using structure-specific enzymes in solution. The cleavage patterns generated by single-strand-specific ribonucleases T1, T2 and A as well as the double-strand-specific cobra venom nuclease (V1) confirm the structure proposed by Stougaard et al. (3) on the basis of the DNA sequence.

The comparison of digests in different buffer systems strengthens the conclusion that the folding of the RNA is insensitive to changes in the use of buffers commonly used for in vitro studies.

The functional center of CopA RNA is loop II where many copy number mutants have been mapped (7). We analyzed two mutant CopA RNAs for structural changes in this region of the molecule. Our data indicate altered loop structures for both mutants.

We have begun to study the structure of the target region of RepA mRNA. Preliminary experiments show that the two guanosines opposite to the tandem cytosines in loop II of CopA RNA are accessible to T1 ribonuclease (data not shown).

MATERIALS AND METHODS

Plasmids.

The following plasmids were used: pKN505 (3), pKN907 (8) and pKN177 (9). Plasmid pKN505 carries the *copA* gene of wild type plasmid R1, whereas pKN907 and pKN177 are miniderivatives of *cop* mutants of plasmid R1. They carry the alleles *copA*-102 and *copA*-104, respectively (7) (cf.fig.5). Plasmid DNA was isolated according to Birnboim and Doly (10) as modified in Maniatis et.al. (11). The *copA* region was cut out from the *copA* mutant plasmids with the restriction endonuclease *Sau*3A. The 262 bp fragment (see fig. 1) was purified on 5% polyacrylamide gels and inserted into the *Bam*HI site of plasmid pBR322 (12) using standard techniques (11). This gave plasmids pGW507 (*copA*-102) and pGW505 (*copA*-104), respectively.

Preparation of 5'-end-labelled *CopA* RNA.

5'-end labelled *CopA* RNA was synthesized in vitro using pKN505 plasmid DNA in modified polymix buffer (13; R.Fast, personal communication). An incubation mixture contained in 100 μ l: 5 mM phosphate buffer pH 7.4, 5 mM $Mg(OAc)_2$, 5 mM NH_4Cl , 2 mM spermidine, 28 mM putrescine, 2 mM dithiothreitol (DTT), 100 μ M each of UTP, GTP and CTP, 13 μ M ATP, 100-400 μ Ci ^{32}P -ATP (New England Nuclear, 3000 Ci/mmol), 2 mM phosphoenol pyruvate (Sigma), 3 μ g/ml pyruvate kinase (Sigma), 15 units RNAsin (Promega Biotech), 4-8 units of *Escherichia coli* RNA polymerase (P-L Biochemicals), and 3-6 μ g of plasmid DNA. After incubation for 55 min at 37°C, RNase-free DNase (Sigma) was added to 20 μ g/ml for an additional 5 min at 37°. 300 μ l of TE-buffer (10 mM Tris-Cl pH 7.9, 1 mM Na_2EDTA) was added on ice. The mixture was extracted twice with an equal volume of phenol/-chloroform, and the upper phase precipitated with ethanol after addition of NH_4OAc to 0.7 M and bulk tRNA to 20 μ g/ml.

The radiolabelled *CopA* RNA was chromatographed on a 6% polyacrylamide gel containing 7 M Urea. The RNA band was cut out after autoradiography. The RNA was eluted by vigorously shaking the gel piece in 0.5 ml of elution buffer (0.1 M $NaOAc$ pH 5.7, 10 mM Na_2EDTA , 0.5% SDS) at room temperature for 1 hr. The radioactivity was determined by counting Cerenkov radiation in a Beckman LS 3801 scintillation counter. The yield was typically higher than 90%. Carrier tRNA was added, the solution extracted twice with phenol/chloroform, once with chloroform, and the upper phase precipitated twice with 0.3 M $NaOAc$ (pH 6.5) and 2 vol of ethanol at -70° for 15 min. After centrifugation the pellet was washed two more times to remove traces of SDS, dried and finally dissolved in TMN-

buffer (20 mM Tris-OAc pH 7.5, 10 mM Mg(OAc)₂, 100 mM NaCl). CopA RNA was renatured by heating to 80° for 4 min followed by slow cooling to room temperature. The RNA was stored at -20°.

Partial enzyme digests for structure analysis.

Enzyme digests were performed in TMN-buffer for 30 min on ice. Reaction volumes were 4.5 µl, and carrier tRNA was present at 70 µg/ml. The concentrations of the individual nucleases, T1 and V1 (P-L Biochemicals), A (Worthington) and T2 (Sigma) needed for appropriate partial digests were determined for each RNA preparation and enzyme batch. The concentration ranges tested were 5×10^{-4} - 0.1 U (units)/µg RNA (T1 and T2), 1×10^{-6} - 1×10^{-3} U/µg RNA (A) and 1×10^{-3} - 0.5 U/µg RNA (V1). Enzyme dilutions were done in TMN-buffer. The reactions were stopped by the addition of 4.5 µl stop solution (9M Urea, 20 mM Na₂EDTA, Xylene Cyanol 0.05%, Bromophenol Blue 0.05%) and heating to 100° for 3 min before placing on ice.

Partial digests in different buffers.

Labelled RNA was isolated as described above. Finally the RNA was dissolved in water. One fifth volume 5x concentrated buffer of either Polymix (13), TMN-buffer (see above) or TMK-buffer (20 mM Tris-OAc pH 7.5, 14 mM Mg(OAc)₂, 60 mM KOAc) was added. The RNA was renatured as above. For digestions, the enzymes were diluted in the respective 1 x buffers prior to addition to the RNA.

Digestions to produce markers.

CopA RNA was digested with ribonuclease T1 under denaturing conditions as described by Wrede et al. (14).

Alkaline ladders were made by incubating CopA RNA in 50 mM NaOH, 1 mM Na₂EDTA at 100° for 15 sec. The reaction was stopped by the addition of an equal volume of neutralization buffer (9.5 M Urea, 50 mM NaOAc, pH 4.5) after which the samples were placed on ice.

Gel electrophoresis and autoradiography.

Samples were run on 12 or 20% polyacrylamide gels containing 7 M Urea. The gels were cast with a wedge such that the thickness increased from 0.5 mm (top) to 1 mm (bottom) and run in a Poker Face Gel Apparatus (Hoefer Instruments) at 65° with constant power setting at 70 W.

Samples were heated to 100° for 3 min prior to loading. Usually aliquots were applied after different times for easier identification of fragment bands in all regions of the RNA. Autoradiography was at -70° using Kodak X-AR5 film and Dupont intensifying screens.

Figure 1. Basic replicon of plasmid R1. The cleavage sites for restriction enzymes PstI (P) and Sau3A (S) are indicated. The leftward PstI site is at position -466 in the sequence in (31). A summary of the components involved in R1 replication control has been published elsewhere (1).

RESULTS

Partial digestion of wild type CopA RNA with different ribonucleases.

CopA RNA was synthesized *in vitro* and subjected to partial digestion ($\ll 1$ cut/molecule) with the following ribonucleases: the single-strand-specific nucleases A, T1 and T2 and the double-strand-specific cobra venom nuclease (V1). Typical digestion patterns are presented in fig.2. In all cases a limited number of cuts were obtained. The results from several such analyses have been summarized in fig.3.

Partial digestions with ribonuclease T1. Ribonuclease T1 has a clear preference for guanosines in non-base-paired regions. The two most prominent cleavages occur after G₁₂ in loop I and G₅₇ in loop II (fig.2). Two other easily accessible residues are the tandem G's at positions 23 and 24. There is weaker, but reproducible cutting also at the remaining four guanosines in the spacer region, G₃₀ and G's 32-34. The most 3'-proximal G₈₈ is cut inefficiently, and at higher enzyme concentrations the cleavage is masked by the more prominent cuts closer to the labelled 5'-nucleotide. The T1 cleavage pattern suggests that at least parts of the loops and the spacer region are non-hydrogen-bonded. No cleavages at guanine nucleotides are visible in the stems except under condition when more than half of the CopA molecules are digested. The weak bands appearing under these conditions correspond to the bonds between G₆₆-A₆₇, A₆₇-A₆₈, G₇₉-G₈₀ and G₈₀-G₈₁ (data not shown). We have no explanation for the cleavage at the adenosine residue.

Partial digestions with ribonuclease A. Ribonuclease A has a strong preference for pyrimidines in non-base-paired regions of RNA. Fig. 2 shows that the enzyme cleaves predominantly at three nucleotides, U₁₁ in loop I and C₂₂ and U₂₇ in the spacer region. Two other strong cleavages occur after

Figure 2. Limited digests of CopA RNA with nucleases V_1 , T_1 , T_2 and A . The units of enzymes used in a standard limited digest (see Methods) were $1.5 \times 10^{-2}U$ (T_1), $1.9 \times 10^{-3}U$ (T_2), $5 \times 10^{-5}U$ (A) and $7 \times 10^{-2}U$ (V_1). A 20% polyacrylamide/7M Urea gel was run with sequential loading of the

samples (left and right section). The numbers and letters indicate the bases counted from the 5'-terminal A in the CopA RNA sequence. L: alkaline ladder.(-): minus enzyme control.

U₁₀ and U₂₅ (fig.2). The weaker cuts in the spacer region include all pyrimidines present. Loop II is cleaved 3' to the three cytosines 56, 58 and 59. Interestingly, we see cleavages of the bonds U₅₂-A₅₃, U₆₄-U₆₅ and U₆₅-G₆₆. These nucleotides are located around the bulged-out U₅₂. We infer that these residues are either not base-paired in all or a subpopulation of the molecules, or that there is sufficient "breathing" in this region of the RNA to become a cleavage site for ribonuclease A. The two pyrimidines at positions 49 and 50 opposite to a bulged-out adenosine are not cleaved.

Partial digestions with ribonuclease T2. T2 is a single-strand-specific nuclease capable of cleaving any of the four nucleotides. Probing of CopA RNA with T2 identifies the two loops, the spacer region and the 3'-tail of the molecule as accessible to nuclease attack. The preferred sites are in the smaller loop, positions A₈, A₉, U₁₀ and U₁₁ (fig.2, see also fig.4). The cleavage after A₈ suggests that this residue is non-base-paired, at least to some extent. T2 cleaves all bonds in the spacer region to various degrees, starting from A₂₁ to G₃₄. In loop II the bond between C₅₆ and G₅₇ is cut predominantly, but cutting also occurs after G₅₇, C₅₈, C₅₉ and, to a lesser extent, A₆₀ and A₆₁. The 3'-tail is attacked at five consecutive nucleotides. Stems I and II are protected from T2 digestion, with the exception of occasional cleavages at G's 80 and 81 and at C₅₄ close to loop II. The reason for this is not understood. Experiments using lower enzyme concentrations or shorter incubation times suggest that the variable activity at these sites is not due to secondary cuts.

Partial digestions with cobra venom nuclease V1. V1 nuclease has been previously used for the identification of nucleotides in helical regions (15,16). Probing of CopA RNA with this enzyme yields strong asymmetric cleavages in the 5'-half of stem I, but also some residues on the opposing strand are cleaved, C₁₇-U₂₀. The longer stem II is also cut mostly on the 5'-side. The main cleavage positions range from G₄₀ to the bond between C₅₄-U₅₅. The bulge U₅₂-A₅₃ bond is not or very weakly cleaved. We see only few cleavages on the 3'-half of stem II, all located close to the 3'-tail. In addition, we find weak, but reproducible cleavages from A₂₁-U₂₇, possibly suggesting that part of the spacer region may be involved in tertiary interactions. No cutting is seen in either of the two loops.

Figure 3. Summary of partial digestion data from the experiment in Fig.2 and about 10 similar analyses. The structure is drawn as in (3) and the cleavage positions are shown. $\blacktriangle$ -V1 ribonuclease, $\blacksquare$ -ribonuclease A, $\bullet$ -ribonuclease T2, $\blacktriangle$ -ribonuclease T1. Preferred cleavage positions are indicated by larger symbols. Parentheses at the 3'-terminal U's indicate heterogeneous CopA RNA termination.

Structure of CopA RNA in different buffers.

Buffer conditions can critically affect structures of biologically active molecules. It is therefore important to control whether a proposed structure exists under conditions where the molecule in question is active. The choice of the buffers TMN, TMK and Polymix was made for the following reasons. TMN buffer or buffers of very similar composition have been used for studies on RNA secondary structure (5). Specific RNA-RNA interactions have been assayed in this buffer (5, 17, and G. Wagner, unpublished data). A TMK buffer is used in coupled transcription-translation systems (18), and we observe very efficient inhibition of RepA expression by CopA RNA in such an *in vitro* system (data not shown). The Polymix buffer has been used extensively in studies of translational fidelity, where the preservation of *in vivo*-like RNA conformations ought to be essential (13, 19). Comparing partial digests of CopA RNA we find approximately the same cleavage patterns for all three buffers tested (fig.4). The cleavages with both T1 and T2 are almost identical. The difference in intensities at the U₂₇ position does not reflect a major

Figure 4. Limited digests in different buffers. CopA RNA renatured in TMN-buffer, TMK-buffer or Polymix (Methods) was digested with either 1×10^{-2} U T1, 7×10^{-2} U V1 or 1×10^{-3} U T2. L: alkaline ladders. Samples on the right were loaded when the Bromophenol Blue dye of the left hand samples had migrated 2/3 of the way to the bottom of the gel. The polyacrylamide gel was 12% with 7M Urea. Numbers 1,2,3 refer to digests in TMN-buffer, TMK-buffer or Polymix, respectively. The numbers refer to bases counted from the 5'-terminal A in CopA RNA. Some residues are given for orientation.

difference in cleavage, since we see more breakage of this bond in Polymix and TMN than in TMK-buffer even in the absence of enzyme. For V1 the digests also have very similar banding patterns, but here some differences can be seen. Cleavages at C₃₇₋₃₉ are seen in TMK- and TMN-buffer, but not in Polymix. Also the bonds G₇₉-G₈₀ and G₈₀-G₈₁ are cut less in Polymix. Cuts

at the end of stem II including U₈₃ and U₈₄ are less pronounced in TMK-buffer. These differences do not change the proposed structure (fig 3), but may rather reflect an influence of the different ionic conditions on the cleavage activity of V1.

Loop structures of in CopA RNA from copy mutants.

Mutations that change the primary nucleotide sequence of CopA RNA and which affect the copy number of plasmid R1 are mostly located in or close to the loop II (7). Changes from G or C to A or U have been found to raise the copy number compared to that of the parent plasmid, perhaps because of the lower binding energy between CopA RNA and its target RNA. Two mutations, originally found in plasmids pKN102 and pKN104, confer a temperature-dependent copy number effect on their plasmids. In contrast to the temperature-independent mutations, CopA RNAs 102 and 104 have base changes that are expected to result in structural alterations around loop II. CopA RNA₁₀₂ has a U in position 54 as compared to a C in the wild type. The consequence is a possible G-U base pair neighboured by two A-U pairs. According to Tinoco et al. (20) the free energy change for these three base pairs in the wild type is -4.4 kcal, whereas ± 0 kcal are expected for the mutant. This could account for a considerable destabilization of the upper stem. CopA RNA₁₀₄ has a U in position 56, such that another base pair could be formed with A₆₁, resulting in a four-base loop. Six-base loops are generally favoured over four-base loops (20). Therefore the copy number decrease of the mutant at higher temperature might reflect an opening up of the U-A bond leading to a wild type-like loop. A corresponding change is also expected for the target RNA structure.

We used T2 nuclease in order to study the structures of the two mutated CopA RNAs and compare them to wild type CopA RNA under identical conditions. In addition to the standard protocol (see Methods) we also probed the RNAs at 37° with varying concentrations of the enzyme. The result can be seen in fig. 5. The most striking observation is that the mutation in the upper stem of mutant 102 renders the molecule accessible to T2 nuclease attack in a region extending far into the stem. Both at 0° and at 37° all bases from C₅₀ to A₆₂ are cleaved. In addition, the degree of cutting is greatly enhanced at the higher temperature, even though the overall degree of digestion is identical (fig. 5 and data not shown). Thus CopA RNA₁₀₂ has an extended single-stranded loop II with higher nuclease accessibility especially at the higher temperature.

Figure 5. Analysis of loop II structures in wild type and mutant CopA RNAs by limited T2 nuclease digestion. CopA RNA from wild type R1 and from copy number mutants pKN102 and pKN104 (see Methods) were probed with 2×10^{-3} U T2 for 30 min. at 0° or at 4×10^{-4} U for 10 min. at 37°. The 12% polyacrylamide/7M Urea gels were run until the Xylene Cyanol dye had migrated to the front of the gel. The nucleotide sequence of the wild type RNA is indicated next to the gel, and base changes in the mutants are shown in the lower diagram. L: alkaline ladder, T₁: limited digest of wild type RNA with T1 ribonuclease as in fig. 2.

CopA RNA₁₀₄ displays cleavages at four consecutive nucleotides, from the mutated U₅₆ to C₅₉. No major differences are seen at the two temperatures, however a slight increase in cleavage at U₅₆ can be seen compared to cleavages at other residues. It is important to note that the cutting at U₅₆ is very much weaker than the cleavages of C₅₆ in the two other CopA RNA species. We feel that this great difference is not explained by a preference of T2 for C residues over U's. For instance, in fig. 2 we see that U₁₂ in loop I is the most prominent cleavage position. U₁₂ is followed by a non-base-paired G on the 3'-side, as is U₅₆. We infer that the low degree of cleavage at the mutated U₅₆ hints at some hydrogen bonding to the opposite A₆₁. Wild type CopA RNA shows identical cleavage patterns at both temperatures.

Miscellaneous.

We have seen that some preparations of CopA RNA show non-enzymatic breakage to a small degree. The break positions are non-random. The preferred bonds broken are U₂₇-A₂₈ > C₂₂-G₂₃ > U₃₁-G₃₂. In order to avoid misinterpretations in the evaluation of the cleavage data we always compared the bands generated by enzymes with the patterns from samples treated identically in the absence of nucleases.

In vitro transcription of CopA RNA results in heterogeneous termination at three consecutive uridines (U₉₁₋₉₃) (data not shown). Therefore, nuclease digestions using the 3'-end-labelled CopA RNA were not feasible.

DISCUSSION

We have probed CopA RNA with various structure-specific ribonucleases in order to determine the secondary structure of the molecule in solution. Knowledge about the stems and loops in the RNA is useful in further studies aiming at the interaction with target RNA sequences that might result in inhibition of RepA protein synthesis in FII plasmids. Our goal is to understand in detail how RNA-RNA interactions via a post-transcriptional mechanism can affect the maintenance of a plasmid at a defined and, in the case of the FII group plasmids, low copy number. In addition, the mechanism by which CopA RNA inhibits the production of RepA protein is of more general interest. Recently, several reports have indicated the involvement of small countertranscripts in down-regulation of protein synthesis. Apart from the FII plasmids, pT181 uses two RNAs whose sequences are complementary and completely contained within the RepC mRNA 5'-leader sequence (21). Here, as suggested for plasmid R100 (22), binding

of the small RNA to its target is believed to alter the accessibility of ribosome binding sites further downstream. Complex formation has not yet been demonstrated. The transposase of Tn10 is also regulated translationally by a countertranscript that starts from the P-out promoter and overlaps with 36 nucleotides of the transposase mRNA in the region of the translation start (23). The small transcript of about 70 nucleotides is expected to have a stable stem-loop structure (24), but is believed to interact with a single-stranded region at the 5'-end of the transposase mRNA (23). Mic RNA controls the translation of OmpF mRNA, presumably by binding to the translation start signals or through affecting the half-life of the mRNA (25). Another example of RNA-RNA interactions leading to a decrease in translation of a gene is found in phage T7. Saite and Richardson (26) find that an RNase III processing-deficient mutant contains an extra stretch of nucleotides at the 3'-end of the mature mRNA of genes 1.1. and 1.2.. This sequence is highly complementary to the Shine and Dalgarno-sequence (27) for gene 1.1., and formation of a double-stranded RNA at that site is supposed to prevent ribosomes from initiating translation. Unfortunately, so far most models for translational inhibition by naturally occurring complementary RNAs have been based solely on hypothetical RNA structures.

In contrast, detailed information exists for the molecules controlling ColE1 replication. Here both the secondary structures of RNAI and the pre-primer/primer as well as complex formation with its effect on primer maturation have been established. Our intention is to follow along these lines by learning about the structure of the components involved in plasmid R1 copy number control and incompatibility.

We report here on the structure of CopA RNA in solution. The cleavage data summarized in fig. 3 establish the secondary structure of the molecule basically as expected from computer predictions. Some points are however noteworthy. First, the loop II where most of the known copy number mutations map (7) is open for base-pairing with the complementary target RNA. Preliminary results show that ribonuclease T1 also cleaves after the two guanines that are located in the complementary loop of the RepA mRNA (data not shown). Therefore, and because of the effect of single base changes in the loop of CopA RNA in several mutants, it is safe to assume that these two loops interact during complex formation. Complex formation is fast and specific in vitro, and CopA RNA is hybridized along the whole length of the molecule (to be published elsewhere).

Second, analyses of the loop structures of two copy mutant RNAs support the conclusion that the stability of the upper stem and the extent of single-strandedness of loop II (and in the target) affect the binding reaction between the two molecules resulting in an altered replication frequency of the parent plasmid. CopA RNA₁₀₄ has a smaller loop II than Cop RNA_{wt}, whereas CopA RNA₁₀₂ has an extended loop due to opening up of the upper part of the helix. Since mutant pKN102 has a higher copy number phenotype, i.e. has a weaker interaction of CopA RNA with the mutant mRNA, in spite of a 6-base loop II sequence identical to the wild type RNA, the structural alteration in the upper stems of CopA and target RNA must alter the presentation of the recognition sequences. More "sloppy" loops may lack the optimal base stacking of the normal recognition site. In a similar way, CopA RNA₁₀₄ with a smaller loop structure is a less efficient inhibitor than the wild type RNA with its structurally more stable 6-base loop.

Third, we find that the bulged-out U₅₂ near loop II is accessible to ribonuclease A. This nucleotide is also attacked by a nuclease from rye nucleus which is specific for non-base-paired structures (Anna Przykorska, personal communication). Bulges in RNA helices have been shown to be located in protein binding domains. Peattie et al.(28) have demonstrated that a highly conserved bulge nucleotide is situated in a region of 5S RNA that is the binding site for ribosomal protein L18. Several other ribosomal and non-ribosomal proteins of pro- and eukaryotic origin likewise bind to RNA helices containing one or two bulge nucleotides (see ref. cited in 28). In addition, accessibility of bulges to nuclease attack has been shown for Escherichia coli 5 S RNA, where the bulged-out nucleotide is flanked by a G-U base pair (15) similar to our results for bulge U₅₂ (see Results). We note that also helix III in RNA1 (ColE1) contains a bulged-out cytosine. The binding reaction of RNA1 to the preprimer is stimulated by the Rop/Rom protein (17,29). So far it is not known whether this region of RNA1 is essential for the Rop/Rom protein's stimulatory effect. Is there any significance to the bulges in CopA RNA? We can conceive of two possibilities. Proteins either of host origin or coded for by plasmid R1 could bind to the RNA and affect complex formation. A possible candidate is the 7kD protein that is encoded by the RNA sequence in front of the RepA reading frame (30). No function has yet been found for this hypothetical protein, but we have recently obtained expression of the gene by fusing the N-terminal part of the 7kD reading frame to LacZ lacking the first 8 amino acids (J.v.Heijne,

personal communication). The other likely explanation for the bulges near loop II is that an imperfect helix in both CopA RNA and the target RNA would drive a reaction towards complex formation. Since the two RNAs are completely complementary, hybrid formation would be favoured over a refolding of the imperfect and, therefore, thermodynamically less stable structure. Experiments are under way to test these possibilities.

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