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Analysis of dominant copy number mutants of the plasmid pMB1

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ABSTRACT

We characterize two dominant copy number mutants of a derivative of plasmid pMB1. One of the two mutations maps in the -35 region of the primer promoter and results in increased promoter activity. The analysis of the secondary structure in the proximity of the mutant sequence suggests a possible mechanism which could be the basis of the promoter-up phenotype. By comparing the properties of the mutant and the wild type plasmid in an in vitro system, we confirm that the primer and not its coding sequence is the target of RNA I inhibition. The second mutation affects the sequence of the primer so that it is less sensitive to inhibition by RNA I. We propose that this mutation stabilizes a secondary structure necessary for primer formation.

INTRODUCTION

The initiation of DNA replication of small plasmids of the ColE1 family depends on the synthesis and maturation of a RNA transcript which serves as primer for DNA synthesis by the enzyme DNA polymerase 1. The precursor of the primer is initiated by RNA polymerase 550 nucleotides upstream from the origin (1). This transcript, that terminates at different sites beyond the origin, is processed by the enzyme RNaseH to yield, in vitro, a 550 nucleotide RNA whose 3' end coincides with the origin of DNA replication. The in vivo processing pattern has not been worked out yet.

A second RNA of 108 nucleotides (RNA I) is a negative regulator of DNA replication (2,3) and in vitro prevents the maturation of the primer (4). Genetic data have established that RNA I regulation occurs via binding of a limited number of bases of two of its three loops with the complementary bases either in the DNA coding sequence or in the primer precursor

(5,6). Tomizawa (7) has shown that RNA I binds to the the primer *in vitro* via a two steps interaction that involves first the "kissing" of the loops of the two RNA molecules and then a more stable interaction which requires the pairing of the 5' single stranded tail of RNA I with the complementary sequence in the primer precursor. Tomizawa has also proposed that this interaction is the basis of the inhibition mechanism.

A second inhibitor, a small protein of 63 amino acids (Rop), participates in the control of plasmid copy number (8,9). *In vitro* Rop stabilizes the interaction between RNA I and the primer precursor and inhibits the formation of the primer (10,11,12).

Here we describe the characterization of two plasmid copy number mutants that are purely dominant, i.e. their genetic defect cannot be complemented *in trans* and they are still able to synthesize an RNA I that is active on a wild type target. Their analysis is consistent with the model described above and contributes data which support the extension of its validity to the molecular mechanism which control plasmid copy number *in vivo*. Furthermore, the analysis of one of the base changes which results in a high copy number phenotype suggests a possible mechanism by which a cruciform structure present at the primer promoter might play a role in plasmid replication.

MATERIALS AND METHODS

Isolation of Mutants

N-Methyl-N-Nitro-N-nitrosoguanidine mutagenesis was as described in Castagnoli et al. (22). The isolation and preliminary characterization of the dominant mutants has been reported (6).

Bacterial Strains, Phages and Plasmids

71/18 is *E.coli* K12Δ(lac pro) [F'lacI^q*lacZ*ΔM15*pro*+] (23). DH1 is *E.coli* K12 F^- *rec*A1 *end*A1 *gyr*A96 *thi*1 *hsd*R17 *sup*E44. The construction of the fusion phage ϕBG34 has already been described (8). ϕBG34*svir*017 was constructed in a similar way using pacI29*svir*017 as the source of the *Hae*III fragment containing the primer promoter. Quantitation of β-galactosidase synthesis was essentially as described by Miller (24).

pEMBL18svir017 was constructed by marker rescue from an 84 base pair fragment carrying the svir017 mutation. 30µg of pacl29 svir017 were digested by Sau3A and the 84 base pair fragment containing the primer promoter was purified by electrophoresis on an 8% polyacrylamide gel. Approximately 3 picomoles of the purified fragment were annealed to one picomole of single stranded pEMBL18 DNA by heating at 90°C and cooling down slowly to room temperature. The annealing reaction was carried out in a volume of 9µl containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl and 1mM DTT. The partially double stranded molecules were completed by adding 10.5 µl of 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each of the four dNTPs, 1 mM ATP, 0.4 units of DNA polymerase I Klenow subunit, 5 units of T4 ligase and by incubating for 10 hours at room temperature. The mixture was then treated with 10 units of S1 nuclease to digest single stranded molecules and then used to transform E.coli 71/18. The transformation was plated on L plates containing 10µg/ml of ampicillin and 300 resistant colonies were picked and tranferred to BA85/20 Schleicher and Schull filters. The filters were then hybridized to 10 pmoles of a 16 nucleotides synthetic oligonucleotide (AAAAGGATTTCAAGAA), labelled with 4 pmoles of [^{32}P] γ ATP (5µC/pmole). Four colonies were positive in the hybridization and all four proved to have lost the Sau3A site at position -32 from the start of primer transcription.

In Vitro Replication

Bacterial extracts which support ColE1 replication were prepared as described by Staudenbauer (1976). The assays were carried out in a volume of 50µl containing 20 µl of the cell extract, 40 mM Hepes-KOH pH8, 100 mM KCl, 10 mM Mg-Acetate, 2mM ATP, 0,4mM each of CTP, GTP, UTP, 0.025 mM each of dATP, dGTP, dCTP and [^{3}H] TTP (1250 cpm/µmole), 0.05 mM cAMP. The incubation is carried out at 30°C for 60 minutes and stopped by addition of 2ml of 10% cold TCA. Acid insoluble radioactivity was counted in a Beckman scintillation counter.

Sequence Analysis

The DNA sequences of the pacl29svir mutants were obtained by the chemical degradation method of Maxam and Gilbert (26).

RESULTS

Isolation of Dominant Mutants

We have recently described the characterization of a large number of mutants in the target of the small RNA (RNA I) which negatively regulates the initiation of DNA replication of the plasmid pMB1 (5,6). Since the target of this molecule overlaps with its coding sequence (13), most of these mutants synthesize an RNA I which is not able to interact with the wild type target. A small number of them (5 out of 120), however, has a phenotype that indicates that they are still able to inhibit the replication of the wild type plasmid. These mutants have been isolated and characterized by using the phasmid system which facilitates the analysis of plasmid replication mutants. It is based on a plasmid λ hybrid that, because of the presence of a second active replication origin (the one from the plasmid), can form plaques on a bacterial host which synthesizes λ repressor. When plasmid replication is inhibited however no plaque is formed. This property allowed us to easily test the sensitivity of a given target to inhibitors of different genotypes by infecting different lysogenic hosts harbouring plasmid replication mutants with a phasmid containing the target for the plasmid replication inhibitor.

Characterization of the Mutants

Five of the mutants isolated (svir105, svir031, svir042, svir017 and svir002) were able to support phasmid growth in a lysogen containing a wild type plasmid and also maintained the ability to inhibit wild type plasmid replication. This indicates that the mutation has hit the target of the inhibitor without affecting the properties of the inhibitor itself. In the plasmid form, three of these mutants (svir105, svir002 and svir017) confer on the host a resistance to a concentration of ampicillin that is higher than the wild type one (Table 1) suggesting that these alterations cause an increase of plasmid copy number. We have confirmed this conclusion by estimating plasmid DNA concentration on agarose gel. The remaining two mutants have no increase in plasmid copy number. Since, as described below, svir017 carries a base change identical to the one present in the ColDF13 derivative cop-1 (14) which is a temperature

Table I. Relative plasmid copy number (1)

Plasmid	copy number
pacl29 w.t.	1
svir002	3
svir017	3.5
svir 105	2

(1) Relative plasmid copy number was estimated by measuring the ampicillin concentration necessary to decrease plating efficiency by a factor of 10 (26). In the case of the wild type plasmid pacl29 this concentration corresponds to 1.5 mg/ml.

sensitive runaway mutant, we measured the copy number of our mutants at 30°C and at 42°C. None of the mutants shows a temperature dependence markedly different from wild type.

We have also analyzed the incompatibility properties of svir002 and svir017 by measuring the ability of the mutant plasmids to transform a bacterial strain that already contains a wild type plasmid. Both mutant plasmids are able to establish in a cell already containing a wild type RNA I with an efficiency higher than the corresponding wild type plasmid. In the presence of Rop however, the efficiency of transformation of svir002 drops to wild type levels while svir017 is only marginally affected (Table 2).

Sequence Analysis

Sequence analysis of the mutants that have been previously characterized indicated that a high percentage of the mutants isolated with this technique are located in the DNA sequence which encodes RNA I and the 5' terminus of the RNA primer (6).

We have carried out sequence analysis for the five mutants that show a purely dominant phenotype. In this case however we sequenced the whole region of approximately 600 nucleotides that contains RNA primer coding sequence and 100 bases upstream of the transcription initiation site. In the case of mutants svir031 and svir042 we did not detect any base change. This result is consistent with the finding that svir031 and svir042

Table II. Transformation efficiency

Recipient host / Incoming plasmid	DH1	DH1 [pacl62 Δrop]	DH1 [pacl62]
pacl29 w.t.	100	1	3
svir002	100	20	3
svir017	300	100	70

Transformation efficiencies are expressed as percent of the transformants obtained when the wild type plasmid pacl29 was used to transform strain DH1. 10 ng of plasmid DNA were used to transform 100µl of competent cells. The transformed cells were plated immediately after the heat shock on selective plates containing 100µg/ml of ampicillin. The plasmid pacl62 has been described (5). pacl62 Δrop was obtained by brief treatment with the enzyme Bal31 of pacl62 linearized by cutting inside the rop gene with PvuII

have wild type copy number. These two mutants which probably carry base alterations in phage sequences have not been studied further. In the case of mutant svir105 we have only been able to detect a C to A transversion at position -65 upstream from the primer transcription initiation site. We are currently in the process of determining whether this mutation is responsible for the observed phenotype. The sequences of svir002 and svir017, when compared with wild type, differ only in the nucleotides that are indicated in the Figure 2. Neither of these mutations affect the RNA I coding sequence. The phenotype of svir002 is caused by a C to T transition in the first nucleotide preceding the start of RNA I synthesis while svir017 DNA shows a G to A base change in the -35 region of the primer promoter.

Since the G to A transition identified in svir017 does not affect the sequence of the primer, we wanted to verify whether this mutation was responsible for the high copy number phenotype. This conclusion was validated by transferring the mutation into a different plasmid background. The Sau3A fragment of 84 bases containing the G to A mutation was purified by

electrophoresis on acrylamide gel of a total Sau3A digest of pacl29 svir017. The isolated fragment was annealed to single stranded pEMBL18 (24) plasmid DNA and, after incubation in the presence of the Klenow subunit of DNA polymerase I, complete double stranded molecules were selected by digestion with S1 nuclease. The double stranded molecules were recovered transforming E.coli 71/18. pEMBL18 plasmids carrying the desired mutation were identified by hybridization to an oligonucleotide of 16 bases that had a sequence complementary to the one present in the mutant svir017. Four candidate pEMBL18 svir017 mutants were checked by digestion with Sau3A and confirmed to have lost the Sau3A site at position -32 because of the G to A transition in the GATC tetranucleotide recognized by the restriction enzyme Sau3A. As predicted, the two isolated pEMBL18 svir017 mutants show a threefold higher copy number than the wild type pEMBL18.

Phenotype of the Mutants in Vitro

To confirm the dominant phenotype of our mutants with independent experiments we tested the replication properties of the svir mutants in a bacterial extract which is able to support ColE1 plasmid replication (Table 3). The addition of 1µg of plasmid DNA in standard conditions results in DNA synthesis that is three times more efficient in the case of svir017 than in the case of the wild type plasmid. Furthermore the incorporation of radioactive precursors in the mutant plasmid is less sensitive to the addition of the inhibitor RNA I. However when the experiment is performed in conditions in which total replication in mutant and wild type plasmid is equivalent (proportionally smaller amount of mutant DNA is added to the extract) the sensitivity of the mutant to RNA I is restored to wild type levels. Given the location of the base change of svir017 (primer promoter region) these results are consistent with svir017 being a promoter-up mutant. svir002 relication is not appreciably different from wild type but is much less sensitive to inhibitory concentrations of RNA I. The addition of purified Rop protein to the extract renders svir002 as sensitive as wild type to RNA I inhibition. Furthermore RNA I prepared from extracts of cells containing the mutant plasmids is indistinguishable from the wild type when tested in the in vitro system.

Table III. Replication in bacterial extracts

		[^{3}H] TTP incorporation(%)		
		Extract(-RNA I)		Extract(+RNA I)
plasmid	DNA added(μg)	-Rop	-Rop	+Rop
pacl29	1	100	25	6-3
svir002	1	120	70	3
svir017	1	300	250	90
svir017	0.3	100	30	15-7

[^{3}H]TTP incorporated into acid insoluble form after the addition of 1 μg of wild type DNA to the extract(-) in the standard assay conditions is taken as 100%. This corresponds to approximately 10,000 cpm. The extract(-) was prepared from a culture of E.coli C600. The extract(+) was prepared from a culture of isogenic cells containing the plasmid pAT153. As a consequence the extract (+) contains the inhibitor RNA I while the extract (-) does not (11). Purified Rop was added at a concentration of 5μg/ml. The figures in the Table have been normalized by multiplying by factors that take into account occasional differences of the extracts in their ability to support plasmid replication. These factors have been obtained by testing the ability of each extract in supporting the replication of plasmid RSF1030 which is insensitive to inhibition by RNA I.

These results are consistent with the properties of these mutants in vivo and support the hypothesis that the replication phenotype of svir017 and svir002 is due to a decreased sensitivity to RNA I inhibition.

svir017 is a Promoter-up Mutation

In principle the phenotype of svir017 whose mutation maps in the primer promoter could be explained by supposing that the target of RNA I overlaps the primer promoter region. Alternatively, and more probably, the G to A transition at position -32 could cause a promoter-up mutation which synthesises more primer which in turn titrates the inhibitor RNA I. To test this we have constructed fusions between the promoter of the primer and the β-galactosidase structural gene. This was achieved by inserting the HaeIII fragment containing

Table IV. Promoter activity

Fusion phage	β-galactosidase units no plasmid	+pBR322
132	25	20
ϕBG34 (w.t.)	70	35
ϕBG34 (svir017)	180	50

The figures are averages of three experiments. The construction of the phage ϕBG34 and the details of the assay have been described (8).

the primer promoter into the HindIII site of phage λ 132 (15). An equivalent fusion was constructed which contained the mutant (svir017) primer promoter. β-galactosidase activity measured in strains carrying the two fusions are consistent with the hypothesis of svir017 being a promoter-up mutation. Approximately three times more β-galactosidase is synthesized by E.coli harbouring the svir017 promoter fused to the β-galactosidase structural gene than the wild type promoter (Table 4). Both fusions however are equally sensitive to inhibition in the presence of Rop and RNA I confirming the findings in the in vitro system that the primer synthesized by the mutated promoter is not less sensitive to Rop and RNA I inhibition. We have also compared the efficiency of primer synthesis when svir017 and the wild type plasmids are used as templates in an in vitro transcription system. The results shown in Figure 1 confirm that the primer promoter of svir017 is two to three times more efficient.

DISCUSSION

We have characterized two pMB1 replication mutants that show a decreased sensitivity to inhibition by the regulator

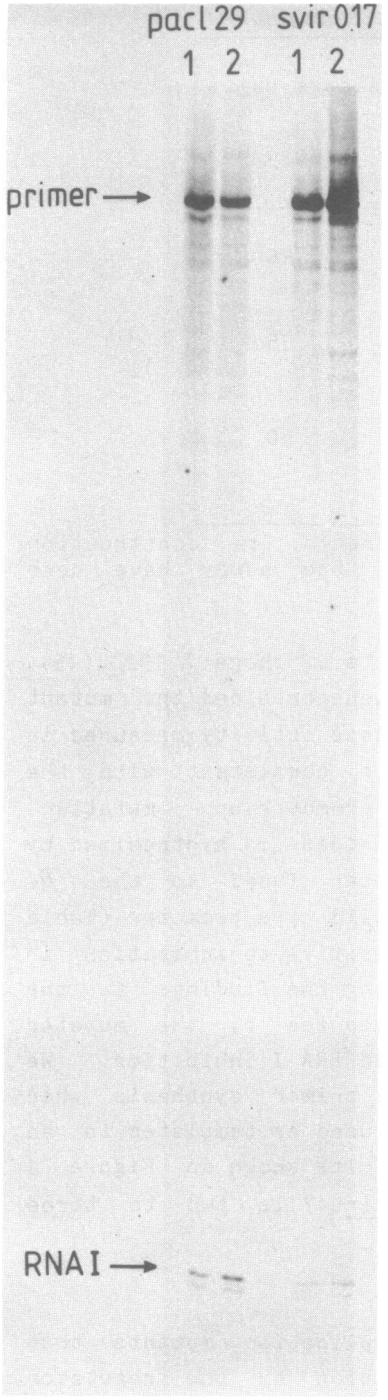

Figure 1. In vitro transcription of plasmid pacl29 and mutant svir017. Supercoiled plasmids (0.35 µg) were incubated for 30' at 30°C in 30 µl of transcription buffer (11) in the presence of 0.35 (lanes 1) or 0.76 (lanes 2) units of RNA polymerase. After digestion with 1.5 units of RNaseH to process the primer, the samples were phenol extracted and electrophoresed on a 6% acrylamide denaturing (8 M urea) gel. The positions where primer and RNA I transcripts migrate are indicated by arrows.

molecule RNA I. As opposed to the majority of mutants that we have characterized so far these two mutants are purely dominant that is they are altered in sequences that do not code for RNA I. As a consequence they synthesize an inhibitor that has wild type properties.

svir017 maps in a region that is 5' of the primer at position -32 from the initiation of primer transcription. The change from G to A in this position results in a promoter-up phenotype. This change does not make the -35 region of svir017 more similar to the consensus sequence that has a C at that position (16). Inspection of the secondary structure of the DNA surrounding the promoter region (Fig. 2) reveals that the -35 box is embedded in a hairpin loop structure. This palindrome is one of the sites where the single stranded specific enzyme S1 linearizes supercoiled pBR322 plasmid (17) indicating that this palindrome does indeed form an hairpin structure in double stranded supercoiled DNA. Since the base that is mutated in svir017 is one of the nine in the stem of this palindrome we could speculate that the alteration present in this mutant could be at the level of promoter secondary structure and not at the level of the primary structure of the -35 region. If linear and cruciform DNA in this region are in equilibrium in the cell, as they seem to be in the test tube, it is evident that the mutation svir017 would move the thermodynamic equilibrium more in the direction of linear helical DNA as opposed to the cruciform structure. If the formation of the hairpin inactivates the promoter this shift in the equilibrium should result in an increase of promoter activity. We are currently testing the hypothesis of the relevance of this secondary structure for promoter function.

The mutation found in svir017 is identical to the one described for cop-1, a ts copy number mutant previously isolated in the plasmid ClodF13 (14). In the context of our plasmid however svir017 does not have a ts phenotype. In this respect it is worth noticing that the palindrome depicted in Figure 2 is reminescent of a terminator structure. It is conceivable that the efficiency of termination of transcripts initiating in neighbouring regions might be affected by the mutation svir017.

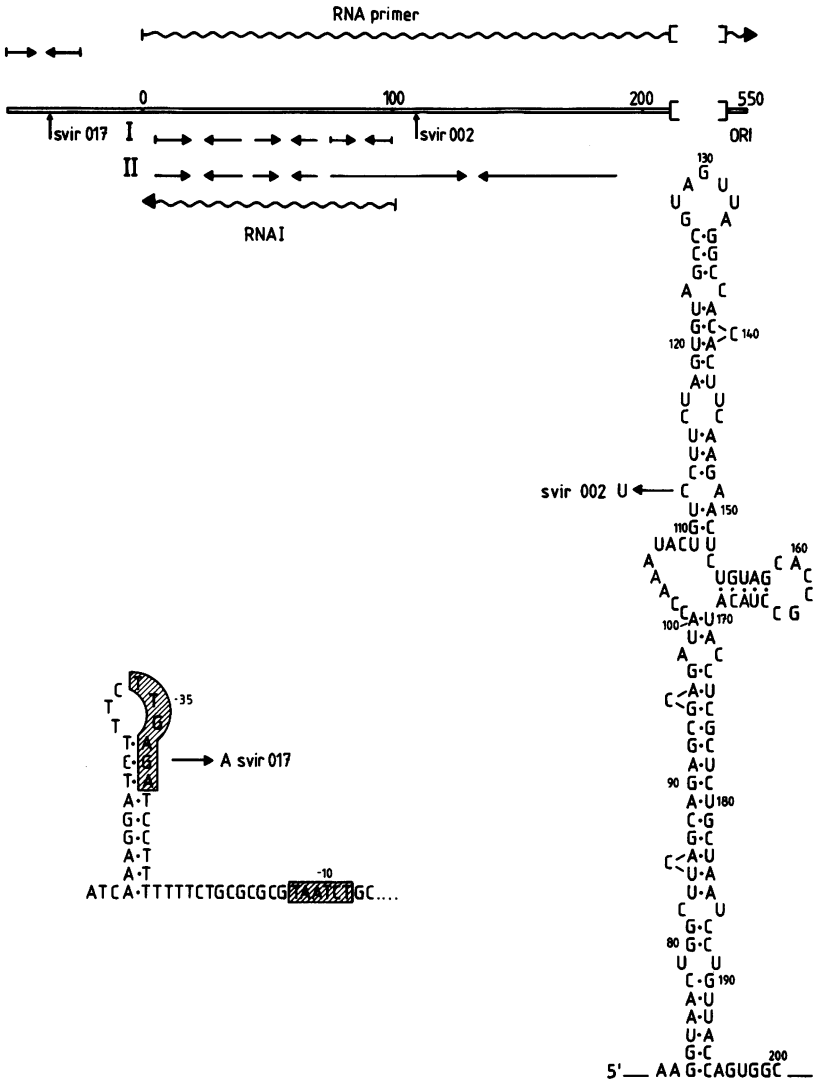

Figure 2. Structural features of the replication origin of plasmid pMB1. The top part of the figure represents the transcripts synthesized from the DNA fragment essential for pMB1 replication. Numbers refer to the distance from the start of primer transcription. Wavy lines represent RNA polymerase transcripts. Inverted repeats which can form alternative structures in the RNA primer (I and II) are indicated by converging arrows. At the bottom of the figure we have depicted two possible secondary structures in the primer promoter region (left) and in the primer transcript (right). The base changes which are responsible for the svir phenotype are indicated.

Furthermore this effect could be temperature dependent. Since transcripts entering the replication origin can serve as primers (18), this could explain the different phenotype of the same mutation in two different contexts.

The isolation of the mutant svir017 allows us to perform experiments in which we study inhibition of plasmid replication by RNA I in conditions in which we can vary the relative amounts of plasmid DNA and RNA primer. This type of experiment allows us to conclude that RNA I is titrated by the RNA primer and not by the corresponding sequence in the plasmid DNA. This provides an independent support to the hypothesis of Tomizawa and Itoh (19) that the primer is the target of RNA I inhibitory activity.

The second mutation (svir002) maps at position +112 from the start of transcription of the primer RNA, i.e. one nucleotide before the start of RNA I. RNA I synthesis is not impaired by this mutation as shown by the ability of the mutant plasmid to inhibit wild type plasmid replication and by the levels of RNA I present in cells harbouring this plasmid. Furthermore we have shown that the replication of this plasmid is less sensitive to inhibition by RNA I. Selzer et al. (20) observed that the first 220 nucleotides of the primer can form two alternative secondary structures (see Fig. 2) and have proposed that structure II is essential for primer maturation and initiation of DNA replication. Structure II is thermodynamically more stable and this model postulates that RNA I inhibits primer formation by binding to the complementary structure in the primer and by stabilizing structure I. The characterization of the mutant svir002 supports this model. In fact the C to T transition at position +112 further stabilyzes the long stem of structure II possibly favouring its formation even in the presence of RNA I and therefore rendering the maturation of the primer less sensitive to inhibition. According to an alternative explanation (illustrated in Fig. 3) the C to T transition at positionn 112 would lower the sensitivity of the primer to RNA I by increasing the length of the stem in Figure 3 thereby hiding in the double stranded structure nucleotide 110 and 111 that are supposed to interact with the 5' single stranded tail of RNA I (7,21). The marked sensitivity of

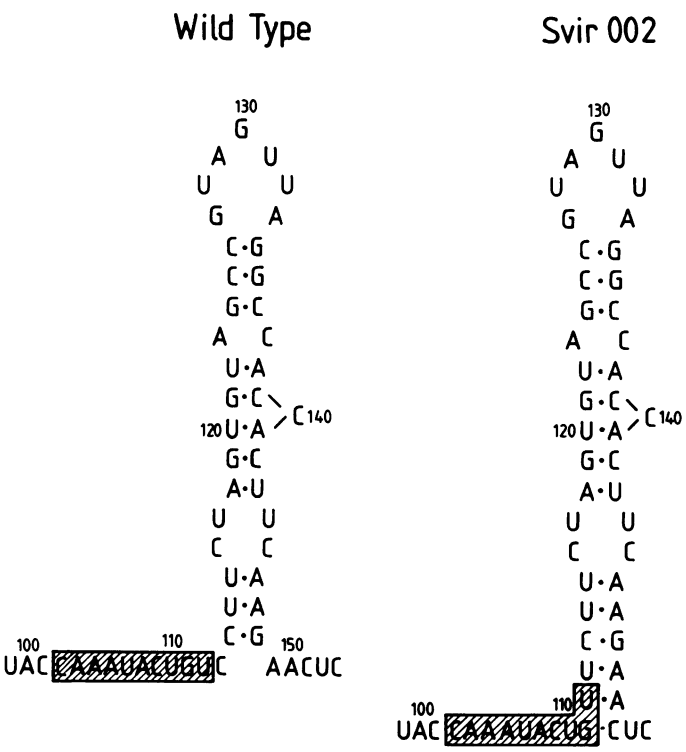

Figure 3. Secondary structure of a portion of the primer transcript. The secondary structure of the primer from nucleotide 99 to nucleotide 153 is shown together with the alteration caused by the mutation svir017. The nucleotides that supposedly are necessary for the stable interaction of RNA I with the primer (7,20) are shaded. Numbers refer to the distance from the start of primer transcription.

svir002 to inhibition by RNA I in the presence of Rop is consistent with both models taking into account the recent findings that Rop cooperates with RNA I in its inhibitory activity (11,12). In the presence of Rop the interaction of RNA I with the RNA primer in conformation I would be preferred and the small variation of free energy favourable to structure II, caused by the mutation svir002, would not be sufficient to shift the equilibrium.

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REFERENCES

1. Itoh, T. and Tomizawa, J. (1980) Proc. Natl. Acad. Sci. USA 77, 2450-2454.
2. Conrad, S.E. and Campbell, J.L. (1979) Role of plasmid-coded RNA and ribonuclease III in plasmid DNA replication. Cell 18, 61-71.
3. Muesing, M., Tamm, J., Shepard, H.M. and Polisky, B. (1981) Cell 24, 235-242.
4. Tomizawa, J., Itoh, T., Selzer, G. and Som, T. (1981) Proc. Natl. Acad. Sci. USA 78, 1421-1425.
5. Lacatena, R.M. and Cesareni, G. (1981) Nature 294, 623-626.
6. Lacatena, R.M. and Cesareni, G. (1983) J. Mol. Biol. 170, 635-640.
7. Tomizawa, J. (1984) Cell 38, 861-870.
8. Cesareni, G., Muesing, M.A. and Polisky, B. (1982) Proc. Natl. Acad. Sci. USA 79, 6313-6317.
9. Som, T. and Tomizawa, J. (1983) Proc. Natl. Acad. Sci. USA 80, 3232-3236.
10. Cesareni, G., Cornelissen, M., Lacatena, R.M. and Castagnoli, L. (1984) EMBO J. 3, 1365-1369.
11. Lacatena, R.M., Banner, D.W., Castagnoli, L. and Cesareni, G. (1984) Cell 37, 1009-1014.
12. Tomizawa, J., Som, T. (1984) Cell 38, 871-878.
13. Cesareni, G. (1981) Mol. Gen. Genet. 184, 40-45.
14. Stuitje, A.S., Spelt, C.E., Veltkamp, E. and Nijkamp, H.J.J. (1981) Nature 290, 264-267.
15. Maurer, R., Meyer, B. and Ptashne, M. (1980) J. Mol. Biol. 139, 147-161.
16. Rosenberg, M. and Court, D. (1979) Ann. Rev. Genet. 13, 319-353.
17. Lilley, D.M. (1980) Proc. Natl. Acad. Sci. USA 77, 6468-6472.
18. Panayotatos, N. (1984) Nucl. Acids Res. 12, 2641-26948.
19. Tomizawa, J. and Itoh, T. (1981) Proc. Natl. Acad. Sci. USA 78, 6096-6100.
20. Selzer, G., Som, T., Itoh, T. and Tomizawa, J. (1983) Cell 32, 119-129.
21. Fitzwalter, T., Tamm, J. and Polisky, B. (1984) J. Mol. Biol. 179, 409-417.
22. Castagnoli, L., Cesareni, G. and Brenner, S. (1982) Genet. Res. Camb. 40, 217-231.
23. Messing, J., Groneborn, B., Muller-Hill, B. and Hofschneider, P.H. (1977) Proc. Natl. Acad. Sci. USA 74, 3642-3646.
24. Miller, J.H. (1972) Experiments in Molecular Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
25. Dente, L., Cesareni, G. and Cortese, R. (1983) Nucl. Acids Res. 11, 1645-1655.
26. Maxam, A. and Gilbert, W. (1980) Meth. Enzymol. 65, 499-560.
27. Uhlin, B.E. and Nordstrom, K. (1977) Plasmid 1, 1-7.