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High-Resolution Crystals and Preliminary X-ray Diffraction Studies of a Catalytic RNA

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Abstract

High-resolution single crystals of a catalytic RNA molecule derived from the sequence of the satellite RNA of tobacco ringspot virus have been obtained. The unit-cell volumes of the RNA crystals vary depending on the crystallization conditions and temperature. The best crystal form, when flash frozen, has space group $P1$ with unit-cell dimensions a = 53.08, b = 71.81, c = 28.03 Å, α = 98.43, β = 104.32 and γ = 74.54°. This form diffracts to a resolution of 2.4 Å. A heavy-atom derivative search is in progress.

Introduction

The satellite RNA of tobacco ringspot virus (STobRV RNA) (Schneider, 1977) is one of many small satellite RNA's of plant viruses. It is an example of an RNA molecule that is spontaneously cleaved to generate the mature RNA genome with a hydroxyl group at the 5′-terminus and a 2′,3′-cyclic phosphate at the 3′-terminus (Cech, 1987; Uhlenbeck, 1987; Sheldon & Symons, 1989). The mature RNA genome of STobRV is 359 nucleotides long but it is known that less than 100 nucleotides are sufficient for effective self-cleavage of a concatenated multigenomic RNA (Buzayan, Gerlach & Bruening, 1986). A 'hammerhead' model has been proposed for the secondary structure of the self-cleaving domain (Forster & Symons, 1987) and it consists of three RNA helices and 13 conserved nucleotides (Fig. 1). Using *in vitro* transcription from a synthetic DNA template derived from the sequence of STobRV, an oligoribonucleotide (69 nucleotides long) was synthesized that can form a structure which contains a self-cleaving domain and a stable long helix to stabilize the cleaved structure (Fig. 1). This molecule, SCR1, cleaves so efficiently that at the end of the transcription incubation reaction, it is found as two fragments in a denaturing gel: a 12-mer and a 57-mer (Fig. 2). In addition, we also constructed a derivative of SCR1, RNA3, which because of a change at position 51 from G to A (Fig. 1) has a greatly reduced rate of cleavage so that the complete 69-mer can be isolated (Fig. 2). Upon prolonged incubation in the presence of magnesium chloride, cleavage will proceed to completion. RNA3 was synthesized and purified in milligram quantities and has been crystallized. The flash-frozen crystals of RNA3 diffract to a resolution of 2.4 Å. This resolution is substantially higher than two previously reported catalytic RNA crystals: crystals of a domain of the group I self-splicing intron from *Tetrahymena thermophila* (2.8 Å, Doudna, Grosshans, Gooding & Kundrot, 1993) and of a hammerhead ribozyme-inhibitor complex (3.2 Å, Pley, Lindes, DeLuca-Flaherty & McKay, 1993).

Methods

Template DNA

Two oligonucleotides were synthesized using an Applied Biosystems 380B DNA Synthesizer. The two oligonucleotides have a complementary overlap of seven bases and these were used in a polymerase chain reaction to generate the double-stranded DNA template used for the *in vitro* transcription reaction (Fig. 1).

Transcription and purification

A transcription reaction contained 5 µg of template DNA and the reagents supplied by Ambion (Austin, USA) in a total volume of 1 ml. An additional 1000 units of T7 RNA polymerase was added [purified by a modification of the protocol described by Wyatt, Chastain & Puglisi (1991)]. The reaction was incubated at 310 K for 1.5 h at which time 100 units of DNase I was added and incubated further for 15 min. The sample underwent phenol/chloroform extraction and ethanol precipitation. To remove unincorporated nucleotides, the sample was filtered through a Microcon 10 unit (Amicon, Inc.). The yield of RNA was about 5 mg ml^{-1} of reaction mixture. The sample was then further purified through a Bio-Rad DEAE SEC DEAE-5PW ion-exchange column (7.5 × 75 mm) on a Waters 650 FPLC system using a salt gradient from 0.4 *M* sodium acetate, 0.1 *M* Tris, 0.2 m*M* EDTA, pH 6.9 to 1.5 *M* sodium acetate, 0.1 *M* Tris, 0.2 m*M* EDTA, pH 7.3 at 277 K, at a flow rate of 0.7 ml min^{-1}. The collected sample was ethanol precip-

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5' ATCGAATTAA TACGACTCAC TATAGGGAGC CCTGTCACCG GATGTGCTTT CCGGTCTGAT GAGTCCGTGA GGACAAAACA GGGCTCCCG  3'
   TAGCTTAATT ATGCTGAGTG ATATCCCTCG GGACAGTGGC CTACACGAAA GGCCAGACTA CTCAGGCACT CCTGTTTTGT CCCGAGGGCT TAA
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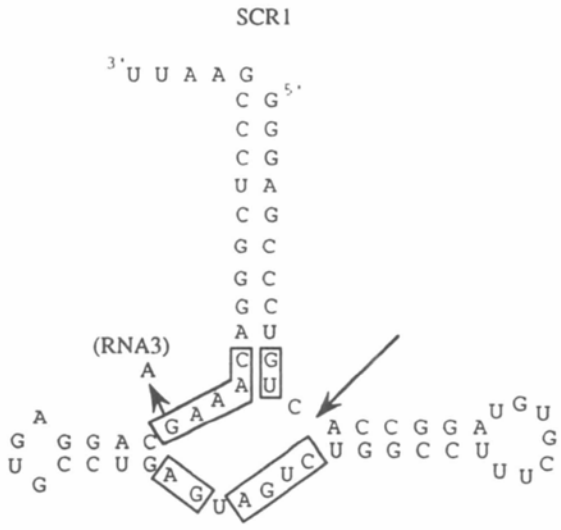

Fig. 1. The secondary structure of self-cleaving RNA's. Nucleotide sequences of SCR1 and RNA3 and the DNA template used to transcribe RNA3 using a T7 transcription system are shown. The long arrow shows the site of cleavage; the short arrow shows the mutation site. The boxed area denotes the conserved self-cleaving domain derived from Hutchins, Rathjen, Forster & Symons (1986).

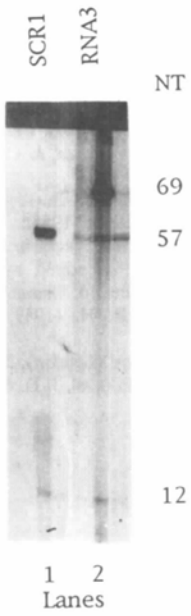

Fig. 2. Cleavage of RNA molecules as detected on a 20% polyacrylamide/7 *M* urea gel and subjected to autoradiography. Lane 1 shows a transcript of SCR1, a derivative (nucleotides 6–58) of the satellite RNA of tobacco ringspot RNA and lane 2 shows a transcript of RNA3 which cleaves less efficiently than SCR1 due to the single mutation of G to A at position 51. NT represents the number of nucleotides.

itated and dried. If ^{32}P-labelled transcript was needed, 5 μCi of [α-^{32}P] CTP was included without changing the total concentration of nucleotides.

Crystallization

Crystallization was performed in a solution containing 0.5 m*M* RNA, 28 m*M* $MgCl_2$, 18.9 m*M* spermine HCl, 35 m*M* Na-cacodylate at pH 6.5 and 6% polyethylene glycol (PEG) 400. This was equilibrated with 18% PEG 400 in 0.08 *M* $MgCl_2$, 0.1 *M* Na-cacodylate at pH 6.5, 18 m*M* spermine HCl by the vapor-diffusion method. The crystals grew within 4 d at room temperature to a size of 500 × 200 × 200 μm. Crystals were sealed in glass capillaries and the data collection was carried out at room temperature or the crystals were flash frozen in the presence of 2-methyl-2,4-pentanediol (MPD) using an Oxford Cryostream liquid nitrogen cooling system manufactured by Stoe Diffraction Systems, and data was collected at 100 K. The reproducibility of the crystals is not very high at present. Crystals have also been obtained in the presence of cadmium or samarium salts, but we have not been able to locate them in the crystals.

Results and discussion

We have been able to obtain reproducibly crystals of a slowly cleaving hammerhead RNA suitable for single-crystal X-ray diffraction analysis by the vapor-diffusion method. Artificial mother liquor was added to the drop and the crystals were mounted in thin-walled glass capillaries. When flash freezing the crystals, MPD was also introduced into the artificial mother liquor prior to the freezing. All crystal forms have the space group *P*1, but the cell parameters varied depending on slight changes in the crystallization conditions and/or the tem-

peratures at which X-ray diffraction experiments were performed. The variation ranges were: a = 52.1–53.08, b = 67.5–72.6, c = 21.9–28.5 Å, α = 98.1–98.6, β = 90.4–104.4, γ = 105.4–105.9°. One or two RNA molecules per unit cell are compatible with the smallest or the largest unit-cell volumes, respectively. Although the larger cells have enough volume to accommodate two molecules, these cells shrink to the smaller unit cells on cooling or flash freezing suggesting a very high solvent content and hence only one molecule per unit cell is deemed most likely.

To test the possibility of the space group being $C2$ rather than $P1$, we converted all unit cells to the $C2$ setting. None of them could be converted into a monoclinic setting. The closest conversion gave α = 91.93 and γ = 90.99°, thus ruling out space group $C2$. The best data set was obtained from the crystals which were flash frozen to 100 K. These crystals diffracted to 2.4 Å and data were collected on a Rigaku rotating-anode X-ray generator run at 50 kV, 100 mA. The image shown in Fig. 3 was taken on an R-AXIS II imaging-plate detector using a graphite monochromator. The unit-cell parameters of the best crystals are a = 53.08, b = 71.81, c = 28.03 Å, α = 98.43, β = 104.32 and γ = 74.54°. Data have also been obtained from crystals which were kept at room temperature. Searches for stable unit cells and heavy-atom derivatives are in progress.

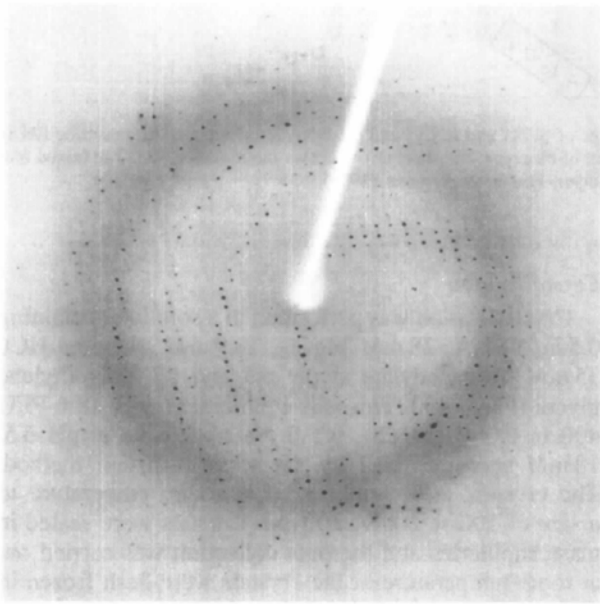

Fig. 3. A 2° rotation diffraction pattern from a flash-frozen crystal of RNA3 at 100 K. The image was taken on an R-AXIS II imaging-plate detector.

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