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Preliminary X-ray diffraction studies of an RNA pseudoknot that inhibits HIV-1 reverse transcriptase

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Abstract

Crystals of a 26-nucleotide pseudoknot RNA, PK26, have been grown. The RNA was produced using phosphoramidite chemistry and was purified by denaturing polyacrylamide electrophoresis. The crystallization was robust with respect to changes in the number of nucleotides and to the salt used as precipitant. The crystals belong to space group $P4_122$ or $P4_322$ with unit-cell dimensions $a = b = 61.6$, $c = 98.9$ Å. The best crystals diffract X-rays to 2.9 Å. Three different sequences incorporating a single 5-bromo-deoxyuridine or 5-bromo-uridine nucleotide were also crystallized. Two of these derivatives are being used to determine the structure by multiple isomorphous replacement.

1. Introduction

RNA pseudoknots have an important function in several biochemical systems (reviewed by Schimmel, 1989; Wyatt, Puglisi & Tinoco, 1989; Draper, 1990; Dam, Pleij & Draper, 1992; Pleij, 1995). Ribosomes contain essential pseudoknots in both 16S rRNA and 23S rRNA. Pseudoknots in mRNA's provide protein binding sites for translation repression and pseudoknots in the 5′ and 3′ untranslated regions of viral RNA's affect mRNA stability and level of translation. The specific amplification of picornavirus RNA in infected cells utilizes pseudoknots. Several retroviruses, including HIV, require a pseudoknot to produce ribosomal frame shifting which is needed for the correct translational products. Pseudoknots are even involved in catalysis, as in the case of the hepatitis delta virus RNA (Perrotta & Been, 1991).

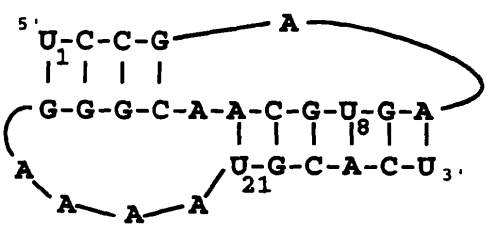
A 26-nucleotide pseudoknot, PK26, specifically inhibits HIV-1 reverse transcriptase with nanomolar affinity (Tuerk, MacDougal & Gold, 1992). This pseudoknot was isolated from an *in vitro* selection for RNA sequences that bind HIV reverse transcriptase. The initial comparative sequence analysis suggested a secondary structure (Model 1, Fig. 1) which was modified in light of additional nucleotide substitution and chemical modification data (Model 2, Fig. 1) (Green *et al.*, 1995). This paper reports the production, purification and crystallization of PK26. The crystals diffract X-rays to 2.9 Å resolution and the structure is currently being determined using two heavy-atom derivatives prepared by chemical synthesis.

2. Materials and methods

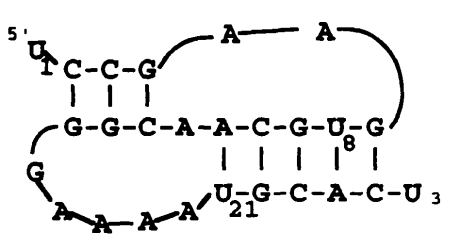
PK26 was synthesized on an Applied Biosystems Model 394 DNA synthesizer. Each preparation of PK26 began with four 1 μmol column syntheses and used phosphoramidite chemistry (Ogilvie, Usman, Nicoghosian & Cedergren, 1988). The same procedures were followed for the synthesis and purification of the unmodified PK26 and for the molecules containing a 5-bromo-deoxyuridine at the first uridine (PK1dBr) or a 5-bromo-uridine nucleotide at the eighth or 21st positions (PK8Br and PK21Br, respectively). Commercially available phosphoramidites (Glen Research) were used to introduce the brominated derivatives. The brominated derivatives were kept in the dark during deprotection, gel electrophoresis and storage to minimize light-induced debromination.

The base and phosphate-protecting groups were removed by incubating the RNA at 328 K overnight in a solution of one part ammonium hydroxide, three parts ethanol. The 2′-hydroxyl protecting groups were removed by (1) lyophilizing the RNA sample to dryness to remove ammonium hydroxide; (2) adding 50 mol of tetra-*n*-butyl ammonium fluoride per mole of base; (3) vortexing the RNA sample at low speed for 1 h in the dark at room temperature; and (4) storing the RNA sample at room temperature in the dark for 24 h. The RNA was then precipitated with sodium acetate/ethanol and resuspended in deionized water. The samples were stored at 253 K until purification.

The deprotected RNA was purified by denaturing polyacrylamide gel electrophoresis (PAGE). Plates with dimensions of 42 × 37 cm and 45 × 37 cm were used with 0.3 cm spacers; these dimensions make a gel with a volume of about 500 ml. Each 20% gel was pre-electrophoresed for 6 h at 45 W. The



Model 1



Model 2

Fig. 1. Proposed secondary structures of PK26.

RNA solution was prepared by adding an equivalent amount of gel loading buffer containing 0.1% bromophenol blue and formamide. Marker lanes contained 0.1% xylene cyanol and 0.1% bromophenol blue. The RNA sample was heated at 338 K for 5 min prior to loading onto the gels. 4–7 mg of crude RNA were loaded onto each gel. Each gel was run at 45–50 W for 12–16 h until the xylene cyanol dye in the marker lanes was within a few centimetres of the bottom of the gel (PK26 migrates slightly slower than xylene cyanol). The RNA was visualized by UV shadowing and the band corresponding to the full-length RNA on each gel was cut out with a sterile razor blade. Each acrylamide band was placed into a sterile 50 ml falcon tube and frozen at 253 K for 2–12 h. Each band was crushed in an autoclaved mortar and pestle and transferred to a 15 ml test tube. The RNA was extracted from the crushed acrylamide by adding 10–15 ml of TEN (10 m*M* Tris pH 7.5, 1 m*M* EDTA pH 8.0, 500 m*M* NaCl) and shaking the tubes for 12 h at 277 K. The acrylamide was separated from the TEN/RNA with a sterile Nalgene 0.2 µm filter unit and the RNA was ethanol precipitated. The purified RNA was resuspended in 1 ml deionized water per gel and desalted and concentrated with successive sterile, deionized water washes in Centricon-3 units (Amicon).

Four 1 µmol synthesis reactions typically yielded 0.5–1 mg of 97–99% pure PK26 (Fig. 2).

Fig. 2. PhosphoImager scan of PK26 before and after purification. Lane 1 is [γ-^{32}P] ATP, lane 2 contains PK26 after deprotection and before preparative PAGE, and lane 3 shows PK26 after the purification. RNA was labeled by polynucleotide kinase with [γ-^{32}P] ATP (New England Nuclear) in a 6 min reaction at 310 K. The samples were run on a sequencing gel at 3400 V for 2 h, exposed to a PhosphoImager screen for 30 min, scanned with a Molecular Dynamics PhosphoImager and quantitated by volume. The sample shown is 99% pure.

The RNA was re-annealed prior to crystallization. A solution of 4 mg ml^{-1} RNA, 10 m*M* sodium cacodylate (pH 6.0) and 5 m*M* magnesium chloride was heated at 338 K for 5 min in a dri-block. The dri-block was removed from the heating element and allowed to cool to room temperature.

X-ray data were collected with a Siemens X100 area detector and Huber four-circle goniostat. A Rigaku RU-200BH rotating-anode X-ray generator with a Huber graphite monochromator operating at 50 kV and 50 mA was used. Crystals were maintained at 100 K in a nitrogen gas stream with an Oxford Cryostream cooler. The data frames were reduced to intensities with *XDS* and *XSCALE* (Kabsch 1988).

Crystals used for the structure determination were stabilized in several steps. All cryostabilizer solutions contained 1.4 *M* sodium acetate, 5 m*M* magnesium chloride and 100 m*M* sodium cacodylate (pH 6.5). The cryoprotectant concentration surrounding the crystals was increased in steps of 1.5–2.5%, allowing a 5 min equilibration period for each step. The crystals were mounted from the cryostabilizer with an ethilon loop attached to a magnetic base (Rodgers, 1994). The crystal was immediately plunged into freshly thawed liquid propane.

3. Results and discussion

Initial crystallization conditions for PK26 were obtained by using conditions that produced crystals of related sequences. A 28-nucleotide RNA (PK28) composed of the PK26 sequence plus two guanosine nucleotides at the 5′ end was produced by *in vitro* transcription (Milligan & Uhlenbeck, 1989; Kundrot, 1996). Lead crystallization conditions were obtained using a sparse matrix (Doudna, Grosshans, Gooding & Kundrot, 1993). The best crystals of PK28 were grown by equilibrating a drop of 1.9 mg ml^{-1} RNA, 0.87 *M* ammonium sulfate, 40 m*M* potassium–MOPS (pH 7.0), 15 m*M* magnesium chloride and 0.25 m*M* spermine against a reservoir of 1.75 *M* ammonium sulfate. Crystals of PK28 diffracted X-rays weakly to 4.6 Å. These conditions also produced crystals of a 29-nucleotide RNA (PK29) produced in the transcription reaction, presumably the so-called 'N + 1 product'. Crystals of PK29 were too small, however, to show X-ray diffraction.

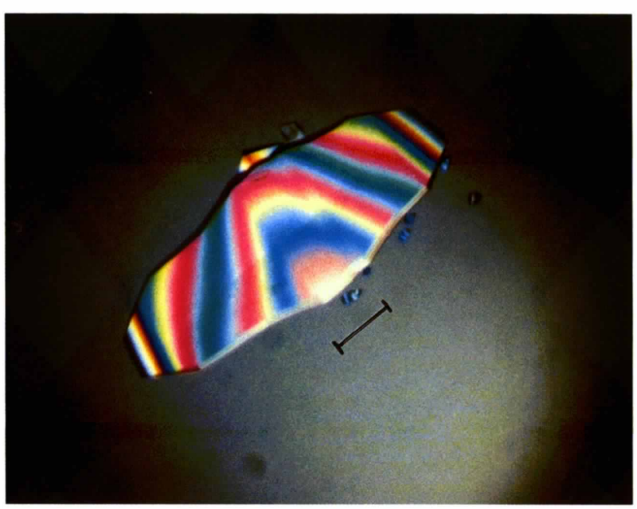


Fig. 3. Crystal of PK26. The bar is 100 µm.

Table 1. *Diffraction limit for different cryostabilizers*

Cryostabilizer*	Soak time (min)	Diffraction limit (Å)
6.5% MPD	5	7.2
10% MPD	5	2.9
6.5% Ethylene glycol	5	5.7
8.0% Ethylene glycol	5	12.0
10% Ethylene glycol	15	7.0
6.5% Glycerol	5	5.1
8.0% Glycerol	5	5.6
18% Glycerol	15	3.6
5% PEG400	5	12.0
8% PEG400	5	10.0
10% PEG400	5	4.0
8% Glucose	15	5.5

*Cryostabilizer solutions contained 1.4 *M* sodium acetate, 100 m*M* sodium cacodylate (pH 6.5), 5 m*M* $MgCl_2$ and the described amount of cryoprotectant.

Surprisingly, the crystallization conditions for PK28 provided the lead conditions for the crystallization of PK26. Moreover, isomorphous crystals were obtained using several different salts as precipitant: ammonium sulfate, lithium sulfate, sodium sulfate and sodium acetate. The useful diffraction limit of the PK26 crystals, however, was dependent on the salt used in the crystallization. Diffuse scatter from crystals grown from ammonium sulfate limited useful diffraction intensities to approximately 5 Å resolution. The diffuse scatter was greatly reduced when the crystals were grown from sodium acetate, sodium sulfate or lithium sulfate. The best crystals of PK26 were grown by equilibrating a drop of 2.0 mg ml^{-1} RNA, 2.5 m*M* magnesium chloride, 55 m*M* sodium cacodylate (pH 6.5), and 0.7 *M* sodium acetate against a reservoir solution of 1.4 *M* sodium acetate and 100 m*M* sodium cacodylate (pH 6.5). Native crystals grew in 7–12 d at room temperature and were typically 600 × 150 × 150 µm in size (Fig. 3). Cryostabilizers containing different concentrations of 2-methyl-2,4-pentanediol, ethylene glycol, glycerol, PEG 400, and glucose were tested (Table 1).

The best diffraction was observed from crystals that were grown using sodium acetate as the precipitant and 10% MPD as the cryoprotectant. The native crystals belong to space group $P4_122$ or $P4_322$ and the PK26 unit-cell dimensions are $a = b = 61.6$ and $c = 98.9$ Å. A native data set with an $R_{sym} = 5.4\%$ for data to 2.9 Å (93% completeness) was collected.

Potential heavy-atom derivatives were obtained by producing and crystallizing PK26 molecules with 5-bromo-uridine nucleotides instead of uridine. The crystals of PK1dBr only grew to a size of 400 × 40 × 40 µm and no X-ray diffraction measurements were attempted from these crystals. Crystals of PK8Br and PK21Br, however, were similar in size to crystals of PK26. Both of these derivatives crystallized in space group $P4_{1/3}22$ with unit-cell dimensions of $a = b = 62.4$, $c = 100.0$ Å and $a = b = 61.8$, $c = 99.2$ Å for PK8Br and PK21Br, respectively. The data from crystals of PK8Br had an $R_{sym} = 11.7\%$ for the data to 3.0 Å (95% completeness). The PK21Br derivative had an $R_{sym} = 7.9$ % to 3.0 Å (99% completeness). The *R* value between native and derivative data over the range 10–4.0 Å is 16.5 and 12.3% for PK8Br and PK21Br, respectively. One heavy-atom site has been located in each of the two heavy-atom derivatives. The sites were identified in difference Patterson and cross-phasing difference Fourier maps. The peak heights of all Harker vectors for each heavy-atom site were $> 2\sigma$. These derivatives are being used to determine the structure by multiple isomorphous replacement.

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