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Crystallization Studies of the Human Immunodeficiency Virus (HIV-1) Tat Protein and its Trans-Activation Response Element (TAR) RNA

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Abstract

Small single crystals are reported of a complex between a small peptide fragment of the HIV-1 Tat protein and a fragment of the RNA to which it binds. Tat is responsible for enhancing the level of expression of the human immunodeficiency virus type 1 (HIV-1) and is a logical target for AIDS therapy. Tat may function to increase the level of transcription initiation or to prevent premature termination of transcripts. *In vitro*, Tat binds through its basic domain (two Lys and six Arg in nine residues) to a three-nucleotide bulge of a stem-loop RNA structure called TAR. Complex formation between Tat and TAR is necessary for Tat activity. Peptides which contain the basic region of Tat also bind to TAR RNA. We have carried out crystallization experiments on a 27-nucleotide fragment of TAR RNA and on complexes between two Tat peptides and TAR.

Introduction

Tat is one of the important regulatory proteins of the human immunodeficiency virus type 1 (HIV-1) which is the virus responsible for the acquired immunodeficiency syndrome (AIDS) (Steffy & Wong-Staal, 1991). Tat functions as a *trans*-activator for the virus: as shown by *in vitro* experiments (Cullen, 1992), it either enhances the rate of transciption initiation or prevents premature termination of viral transcripts. A complex between Tat and an RNA stem-loop structure (TAR) located at the 5′-end of all HIV-1 transcripts is implicated in this function (Cullen, 1992). *In vivo* activity may require additional interactions of the complex with other cellular factors (Gaynor, 1992; Madore & Cullen, 1993). Although the mechanism of action of Tat is not yet clear, both Tat and TAR RNA constitute possible targets for anti-HIV drug therapy. A better understanding of the Tat–TAR interactions through structural studies of the complex is, therefore, essential.

Full-length Tat is an 86-residue protein encoded by two exons (Aldovini *et al.*, 1986). However, only the first exon of Tat (encoding residues 1–72) is necessary for *in vitro* activity. TAR RNA is a 59-nucleotide stem-loop structure (Cullen, 1992). Mutational and competition experiments together with ribonuclease protection assays (Cullen, 1992; Harper & Logsdon, 1991) have mapped the regions of interaction between Tat and TAR RNA. The basic domain of Tat (residues 49–57 with the sequence RKKRRQRRR) and especially the arginine residue at position 52 is essential for high-affinity binding to TAR RNA (Frankel, 1992). The complementary region on the RNA consists of a three-nucleotide bulge with an invariant U at position +23, two short stem regions immediately above and below the bulge, and a six-nucleotide loop which is not involved in binding but is necessary to stabilize the RNA structure (Sumner-Smith *et al.*, 1991). A minimal TAR RNA fragment would typically include nucleotides +18 to +44. Peptides which span the basic region of Tat also bind to TAR RNA with nanomolar affinity (Frankel, 1992) and a high level of specificity (Weeks, Ampe, Schultz, Steitz & Crothers, 1990).

Unlike the successful X-ray studies of tRNA's, few high-resolution crystal structures of RNA oligoribonucleotides have been reported to date (Dock-Bregeon *et al.*, 1989; Holbrook, Cheong, Tinoco & Kim, 1991). In addition, few crystal structure studies of protein–RNA complexes (Cavarelli, Rees, Ruff, Thierry & Moras, 1993; Rould, Perona, Soll & Steitz, 1989; Ruff *et al.*, 1991) have been completed. In the case of TAR RNA, an NMR study of the interactions between arginamide (an arginine analogue) and a TAR RNA fragment has appeared (Puglisi, Tan, Calnan, Frankel & Williamson, 1992). NMR studies of the TAR hairpin loop were also carried out (Colvin, White, Garcia-Blanco & Hoffman, 1993; Jaeger & Tinoco, 1993; Michinicka, Harper & King, 1993). We would like to supplement the information obtained so far with a crystal structure study of the Tat–TAR RNA complex. To this end, we have begun studies on complexes between

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the Tat peptides and TAR RNA. We report here the crystallization of a 27-nucleotide TAR RNA fragment (nucleotides +18 to +44) as well as of a complex between the RNA and a 14-residue basic peptide (RKKRRQRRRPPQGS) spanning residues 49–62 of the Tat protein. The results of the screening experiments for crystallization conditions are presented. The conditions used to obtain single crystals and the properties of the crystals are also reported.

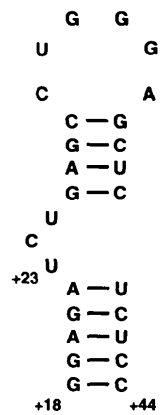
Experimental

Chemicals

The chemicals used for the protein incomplete factorial of Jancarik & Kim (1991) were purchased as a 'Crystal Screen' kit from Hampton Research. A few of the chemicals used in the RNA sparse matrix (Doudna, Grosshans, Gooding & Kundrot, 1993) and our own incomplete factorial for the complex were of the molecular biology grade from Sigma [ammonium sulfate, magnesium chloride (1 *M* solution), sodium chloride, Hepes, Mes, Mops, PEG8000, spermine and spermidine] and from Boehringer-Manheim (Tris). Other chemicals were obtained as ACS grade reagents from Fisher Scientific [barium chloride, cobalt(II) chloride, copper(II) chloride, lithium chloride, nickel(II) chloride and sodium phosphate], Sigma (calcium chloride, cacodylic acid, lithium sulfate, all the polyethylene glycols and Pipes), and Mallinckrodt [chromium(III) chloride]. Organic solvents used in crystallization were from Aldrich (MPD), Fisher (dioxane, *t*-butanol, 1,6-hexanediol and isopropanol) and Quantum Chemicals (ethanol). The water was purified with a Barnstead Nanopure water purifier system.

In vitro transcription and gel purification of the TAR RNA fragment

The TAR RNA fragment (Fig. 1*a*) was prepared by transcription of two templates (see below) with T7 RNA polymerase by a method previously described (Delling *et al.*, 1991). According to this method, equimolar amounts (20 m*M*) of the two oligodeoxyribonucleotide templates labeled NS1 (17 bp, *in vitro* transcription promoter of T7 RNA polymerase, 5′-TAATACGACTCACTATA-3′) and NS10 (44 bp, for *in vitro* transcription of the TAR fragment, 3′-ATTATGCTGAGTGATATCCTCTAGACTCG-GACCCTCGAGAGAGG-5′) were annealed in 0.1 *M* NaCl at 363 K for 5 min. A transcription mixture (2 ml) containing 10 pmol of the annealed templates, 4 m*M* each of rATP, rCTP, rGTP and rUTP (Pharmacia), 10 m*M* $MgCl_2$, 400 units of RNAsin inhibitor (Promega), and approximately 1700 units of T7 RNA polymerase (Stratagene) in reaction buffer (Stratagene) was incubated overnight at 310 K. The reaction buffer consisted of 40 m*M* Tris, pH 8, 2 m*M* spermidine, 30 m*M* DTT, 8 m*M* $MgCl_2$ and 50 m*M* NaCl. A cloudy solution and a white precipitate were obtained at the end of the reaction. RQ1 deoxyribonuclease (20 units, Promega) was then added and the resulting mixture was incubated at 310 K for 15 min. The reaction mixture was aliquoted into four tubes and extracted with 1 volume of phenol–chloroform (Invitrogen). The organic phase was back-extracted once with 600 µl of H_2O. The combined aqueous phases were divided into eight tubes of 400 µl each. Sodium acetate at pH 5.5 was then added to a concentration of 0.33 *M*. The RNA was precipitated with 2.2 volumes of a 1:1 mixture of 95% ethanol–acetone and centrifuged for 15 min to yield pellets which were subsequently washed with 300 µl of 95% ethanol and air dried. All centrifugation steps were carried out at 16000*g* in an Eppendorf centrifuge. Each of the pellets was dissolved in 10 µl of RNAse-free



(*a*)

Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro Lys Thr
20
Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys Gln Val Cys Phe Ile Thr
40
Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln
60
Gly Ser Gln Thr His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
72 80
Pro Thr Gly Pro Lys Glu
86

(*b*)

Fig. 1. (*a*) Predicted (Sumner-Smith *et al.*, 1991) secondary structure of the TAR RNA fragment. The two C·G base pairs at the base of the stem have been reversed to improve the efficiency of transcription (Tan & Frankel, 1992). (*b*) Amino-acid sequence of the Tat protein (Aldovini *et al.*, 1986). The sequence of the 14-mer peptide is underlined.

H_2O. The RNA solutions were combined and then mixed with 1 volume of sequencing grade formamide, incubated at 363 K for 5 min, centrifuged for 5 min and electrophoresed on a 15% polyacrylamide–7 *M* urea gel (290 × 200 × 1.5 mm, Bethesda Research Lab) at 45 mA. The main RNA band on the gel was detected by UV shadowing, excised, and eluted overnight in 20 ml of 0.3 *M* sodium acetate, pH 5.5 on a shaker at 310 K. The gel fragments were separated from the RNA solution by centrifugation. The eluate was divided in 500 μl aliquots in approximately 32 micro-centrifuge tubes, precipitated with 1 ml of a 1:1 mixture of ethanol–acetone and the RNA pellets were recovered by centrifugation for 15 min. Each of the RNA pellets was washed with 300 μl of 95% ethanol and air dried. Each pellet was then dissolved in 10 μl of H_2O, and the solutions were combined and extracted with 1 volume of phenol–chloroform. The organic layer was back-extracted once with 200 μl of H_2O. The combined aqueous phases were aliquoted into two tubes and sodium acetate was added to each to a final concentration of 0.3 *M*. The RNA in each tube was precipitated with 1 ml of a 1:1 mixture of ethanol–acetone and centrifuged for 15 min. Each RNA pellet was washed with 300 μl of 95% ethanol, air dried, and dissolved in 20 μl of H_2O. A 1–5 μl aliquot of the combined RNA solutions was diluted to 0.7–1.0 ml with H_2O and the RNA concentration was measured at 260 nm (Shimadzu spectrophotometer) in a 1 cm path length cell using an extinction coefficient of 37 μg ml^{-1} for 1 AU. The yield of purified RNA for a preparation of this scale was 0.6–1 mg. The unused RNA was lyophilized to dryness in a Savant Speedvac centrifuge and stored at 193 K.

RNA melting curve

RNA for crystallization was dissolved in 10 m*M* K phosphate, pH 7.0, 100 m*M* NaCl, to a concentration of 0.04 mg ml^{-1}. Melting curves were obtained by heating the RNA solution in a Beckman spectrophotometer with temperature controller and monitoring the absorption at 260 nm as a function of temperature. A melting profile is shown in Fig. 2. The melting temperature (T_m) of the TAR RNA fragment was 339 K.

RNA pre-incubation

To promote folding of the RNA for crystallization, RNA solutions at approximately 10 mg ml^{-1} in the appropriate buffer were incubated at 353 K (above the T_m) in a heating block for 15 min. The heating block was then removed from the heating unit and allowed to cool slowly overnight to room temperature (≃295 K). The RNA was then diluted to approximately 5 mg ml^{-1}. Complex formation between the folded RNA and the peptides was achieved by a 30 min incubation of the mixture at room temperature.

Peptide synthesis and purification

The amino-acid sequence of Tat is shown in Fig. 1(*b*). Peptides containing the basic region (residues 49–57) were synthesized by the solid-phase method (UCLA peptide synthesis facility). The peptides were purified by HPLC to better than 99% purity using a preparative reverse-phase C_{18} μBondapak column from Waters (2.5 × 10 cm) with a water–acetonitrile gradient containing 0.05% trifluoroacetic acid (TFA from Pierce). Peptide elution was monitored at 214 nm. The purified peptides were collected in sterile 5 ml cryogenic plastic tubes, frozen in a CO_2–isopropanol bath, and lyophilized to dryness in a Labconco freeze dryer or a Savant Speedvac centrifuge. To remove residual TFA, the lyophilized peptides were re-dissolved in sterile H_2O, re-lyophilized and stored desiccated at 253 K. The yield was 50–60% for 5–10 mg of peptide injected into the HPLC. The amino-acid sequences (RKKRRQRRR-PPQGS for the 14-residue peptide and RKKRRQRRR for the 9-residue peptide) were verified by amino-acid sequencing. The molecular weights (1806 for the 14-residue peptide and 1340 for the nine-residue peptide) were verified by fast atom bombardment mass spectrometry (UCLA mass spectrometry facility). For the crystallization experiments, the peptides were weighed on a Mettler analytical balance to 0.02 mg accuracy and dissolved in sterile H_2O.

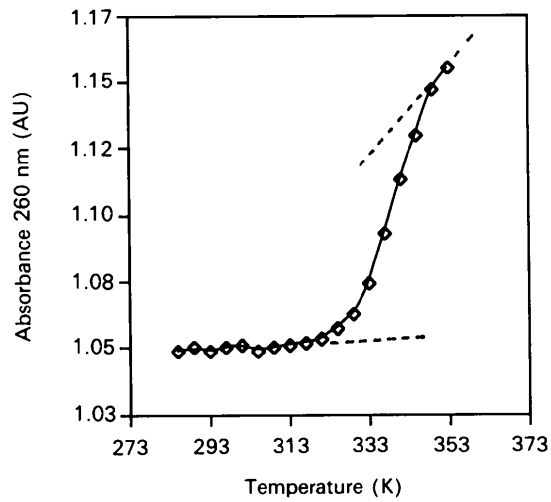


Fig. 2. Melting curve of the TAR RNA fragment using a 1 cm path length cell. The upper and lower baselines (---) are estimated according to the method of Puglisi & Tinoco (1989). The melting temperature (T_m) was the midpoint of the transition and was calculated to be 339 K.

Crystallization

RNA solutions were centrifuged through a 0.2 μm centrifuge filter (Millipore) before use in crystallization experiments. Both the hanging-drop (McPherson, 1985) and the sitting-drop (Stura & Wilson, 1992) vapor-diffusion methods were used for crystallization trials. For RNA solutions, a screen for crystallization conditions was carried out using both a protein sparse matrix with 50 conditions (Jancarik & Kim, 1991) and an RNA sparse matrix with 44 conditions (Doudna *et al.*, 1993) at 277 and 295 K. A total of 1 mg of RNA was required for both screens. For solutions of the peptide–RNA complex, we designed our own screening test of 72 trials using the incomplete factorial method of Carter & Carter (1979) with conditions selected to favor complex formation, namely pH between 6 and 8 and low ionic strength solutions (Table 1). Less than 500 μg of RNA and 100 μg of peptide were required for crystallization trials at 295 K. Crystals were obtained for condition 65 of Table 1.

To protect the RNA from nucleases, sterile plasticware and clean glassware (baked for several hours at 523 K) were used. Solutions were prepared in autoclaved water and sterile-filtered (0.2 μm).

Tissue culture trays with 24 wells were obtained from Linbro (hanging drops) and Costar (sitting drops). The sitting-drop rods were prepared according to the method of Stura & Wilson (1992) and affixed to the trays using vacuum grease. The solutions for crystallization were placed on silanized cover slips (hanging drops) or sitting rods (sitting drops). The initial volume of the drop solutions was 2–3 μl. The volume of the precipitant in the wells was 0.7 (sitting drops) or 1.0 ml (hanging drops).

Characterization of single crystals by gel electrophoresis

Single crystals were characterized by gel electrophoresis using Phast gels from Pharmacia. The crystals were transferred to microhematocrit capillary tubes and washed 3–4 times with reservoir solution. After each wash, the resuspended crystals were centrifuged briefly in a clinical centrifuge equipped with a rotor for hematocrit tubes (International Equipment Co.) and dried with sterile paper wicks (Hampton Research).

For detection of the RNA, a washed crystal was dissolved in 4–5 μl of H_2O and transferred to 20% polyacrylamide gels (Pharmacia Phast system) with gel-loading capillary pipet tips. The gels were 0.45 mm thick and contained 0.112 *M* Tris–acetate at pH 6.5, 7.5% acrylamide–3% Bis (stacking gel), and 20% acrylamide–2% Bis (separating gel). The Phast native buffer strips contained 0.88 *M* L-alanine and 0.25 *M* Tris, pH 8.8 in 2% agarose (IEF grade).

Table 1. *Solution conditions for incomplete factorial crystallization of the peptide–RNA complex*

No.	Buffer (50 m*M*)	Precipitant	Salt (50 m*M*)	Additive (1 m*M*)
1	Na Mops, pH 7	30% PEG 400	LiCl	Spermine
2	Na cacodylate, pH 6	20% PEG 400	$(NH_4)_2SO_4$	
3	Na Hepes, pH 8	20% PEG 600	$MgCl_2$	Spermine
4	Na Mops, pH 7	30% PEG 400	$(NH_4)_2SO_4$	
5	Na Hepes, pH 8	20% PEG 400	Li_2SO_4	Spermine
6	Na cacodylate, pH 6	10% PEG 400	$CaCl_2$	Spermine
7	Na Hepes, pH 8	10% PEG 600	$(NH_4)_2SO_4$	
8	Na cacodylate, pH 6	30% PEG 400	Li_2SO_4	Spermine
9	Na Mops, pH 7	10% PEG 400	Li_2SO_4	
10	Na Mops, pH 7	20% PEG 600	NaCl	
11	Na cacodylate, pH 6	10% PEG 400	$MgCl_2$	
12	Na Hepes, pH 8	10% PEG 600	LiCl	Spermine
13	Na cacodylate, pH 6	30% PEG 600	NaCl	
14	Na Hepes, pH 8	30% PEG 400	NaCl	
15	Na Mops, pH 7	30% PEG 600	$MgCl_2$	Spermine
16	Na Hepes, pH 8	20% PEG 600	$CaCl_2$	Spermine
17	Na cacodylate, pH 6	20% PEG 600	LiCl	
18	Na Mops, pH 7	30% PEG 400	$CaCl_2$	Spermine
19	Na Mops, pH 7	30% PEG 400	$MgCl_2$	
20	Na Hepes, pH 8	10% PEG 400	LiCl	Spermine
21	Na cacodylate, pH 6	20% PEG 400	$MgCl_2$	Spermine
22	Na cacodylate, pH 6	10% PEG 400	$(NH_4)_2SO_4$	
23	Na Mops, pH 7	20% PEG 400	LiCl	
24	Na Hepes, pH 8	20% PEG 400	$CaCl_2$	Spermine
25	Na Mops, pH 7	30% PEG 600	$CaCl_2$	
26	Na Hepes, pH 8	20% PEG 600	Li_2SO_4	
27	Na cacodylate, pH 6	30% PEG 600	Li_2SO_4	Spermine
28	Na Hepes, pH 8	10% PEG 600	NaCl	Spermine
29	Na Mops, pH 7	10% PEG 400	NaCl	Spermine
30	Na cacodylate, pH 6	20% PEG 400	NaCl	
31	Na cacodylate, pH 6	30% PEG 600	LiCl	Spermine
32	Na Hepes, pH 8	10% PEG 600	$MgCl_2$	
33	Na Mops, pH 7	20% PEG 600	$(NH_4)_2SO_4$	Spermine
34	Na Mops, pH 7	10% PEG 600	Li_2SO_4	Spermine
35	Na Hepes, pH 8	30% PEG 600	$(NH_4)_2SO_4$	
36	Na cacodylate, pH 6	10% PEG 600	$CaCl_2$	
37	Na Mops, pH 7	30% PEG 3350	$CaCl_2$	
38	Na Hepes, pH 8	20% PEG 3350	Li_2SO_4	
39	Na cacodylate, pH 6	30% PEG 3350	Li_2SO_4	Spermine
40	Na Hepes, pH 8	10% PEG 3350	NaCl	Spermine
41	Na Mops, pH 7	20% PEG 1000	NaCl	Spermine
42	Na cacodylate, pH 6	20% PEG 1000	NaCl	
43	Na cacodylate, pH 6	30% PEG 3350	LiCl	Spermine
44	Na Hepes, pH 8	10% PEG 3350	$MgCl_2$	
45	Na Mops, pH 7	20% PEG 3350	$(NH_4)_2SO_4$	Spermine
46	Na Mops, pH 7	10% PEG 3350	Li_2SO_4	Spermine
47	Na Hepes, pH 8	30% PEG 3350	$(NH_4)_2SO_4$	
48	Na cacodylate, pH 6	10% PEG 3350	$CaCl_2$	
49	Na Mops, pH 7	30% PEG 1000	LiCl	Spermine
50	Na cacodylate, pH 6	20% PEG 1000	$(NH_4)_2SO_4$	
51	Na Hepes, pH 8	20% PEG 3350	$MgCl_2$	Spermine
52	Na Mops, pH 7	30% PEG 1000	$(NH_4)_2SO_4$	
53	Na Hepes, pH 8	20% PEG 1000	Li_2SO_4	Spermine
54	Na cacodylate, pH 6	10% PEG 1000	$CaCl_2$	Spermine
55	Na Hepes, pH 8	10% PEG 3350	$(NH_4)_2SO_4$	
56	Na cacodylate, pH 6	30% PEG 1000	Li_2SO_4	Spermine
57	Na Mops, pH 7	10% PEG 1000	Li_2SO_4	
58	Na Mops, pH 7	20% PEG 3350	NaCl	
59	Na cacodylate, pH 6	10% PEG 1000	$MgCl_2$	
60	Na Hepes, pH 8	10% PEG 3350	LiCl	
61	Na cacodylate, pH 6	30% PEG 3350	NaCl	
62	Na Hepes, pH 8	30% PEG 1000	NaCl	
63	Na Mops, pH 7	30% PEG 3350	$MgCl_2$	Spermine
64	Na Hepes, pH 8	20% PEG 3350	$CaCl_2$	Spermine
65	Na cacodylate, pH 6	20% PEG 3350	LiCl	
66	Na Mops, pH 7	30% PEG 1000	$CaCl_2$	Spermine
67	Na Mops, pH 7	30% PEG 1000	$MgCl_2$	
68	Na Hepes, pH 8	10% PEG 1000	LiCl	Spermine
69	Na cacodylate, pH 6	20% PEG 1000	$MgCl_2$	Spermine
70	Na cacodylate, pH 6	10% PEG 1000	$(NH_4)_2SO_4$	
71	Na Mops, pH 7	20% PEG 1000	LiCl	
72	Na Hepes, pH 8	20% PEG 1000	$CaCl_2$	Spermine

The programmed separation method was from a protocol by Andersson & Johansson (Pharmacia). After electrophoresis, RNA bands were detected by silver staining (Flensburg & Hagberg, 1987).

For detection of the peptides, a dissolved crystal was transferred to a high-density Phast gel containing 30% ethylene glycol, 7.5% acrylamide–2% Bis in the stacking zone and 20% acrylamide–2% Bis in the separating zone with the same buffer described above. The Phast SDS buffer strips contained 0.2 *M* Tris, pH 8.1, 0.2 *M* tricine and 0.55% SDS in 3% agarose IEF. The programmed separation method used was specific for high-density gels (Pharmacia). After electrophoresis, the peptide band was stained for 24 h at room temperature with a Coomassie blue solution (Pharmacia).

Analysis of crystals by X-ray diffraction

Preliminary analysis of X-ray diffraction was obtained with a Rigaku R-axis II imaging plate equipped with an RU-200 rotating-anode generator for the crystals of the peptide–RNA complex. Crystals of the RNA alone were analyzed with a Nonius precession camera and the same X-ray generator.

Results

Screen for crystallization conditions for a TAR RNA fragment

An initial study of the crystallization conditions for the TAR RNA fragment was carried out using both protein (Jancarik & Kim, 1991) and RNA (Doudna *et al.*, 1993) sparse matrices at 277 and 295 K. The conditions which resulted in birefringent microcrystals for the RNA are shown in Table 2. The initial drop volume for each condition was 2 μl and contained 2–2.5 mg ml^{-1} RNA and half the precipitant concentration used in the well. On the basis of these results, further experiments with PEG3350 (instead of PEG4000) were carried out, ultimately leading to the conditions described in the following section.

Crystallization of a TAR RNA fragment

Birefringent crystals (200 × 150 × 30 μm) as shown in Fig. 3(*a*) were obtained at 295 K by the sitting-drop vapor-diffusion method. The habit suggests that the crystals may be orthorhombic. The drop (2 μl) contained an initial RNA concentration of 2.5 mg ml^{-1} in 50 m*M* Na cacodylate, pH 6, 25 m*M* NaCl, 2.5%(*w*/*v*) PEG3350, and 0.05 m*M* $MgCl_2$ and was equilibrated against a 1 ml well solution of 50 m*M* Na cacodylate, pH 6, 5 m*M* NaCl, and 40%(*w*/*v*) PEG3350. When photographed for 8 h on a precession camera (see *Experimental*), few reflections were visible with the highest resolution being 15 Å.

Table 2. *Results for the protein and RNA sparse matrix crystallization screens for the TAR RNA fragment*

Method*	Conditions	Results
1†	0.1 *M* Na citrate, pH 5.6, 0.2 *M* NH_4 acetate, 30%(*w*/*v*) PEG 4000	Birefringent microcrystals
1†	0.1 *M* Na acetate, pH 4.6, 0.2 *M* NH_4 acetate, 30%(*w*/*v*) PEG 4000	Birefringent microcrystals
1†	0.1 *M* Na cacodylate, pH 6.5, 0.2 *M* Na acetate, 30%(*w*/*v*) PEG 8000	Birefringent microcrystals
1†	0.1 *M* Na citrate, pH 5.6, 20%(*v*/*v*) 2-propanol, 20%(*w*/*v*) PEG 4000	Birefringent microcrystals
1†	0.1 *M* Na acetate, pH 4.6, 8% PEG 4000	Birefringent microcrystals‡
2†	0.5 *M* K succinate, pH 5.5, 3 *M* $(NH_4)_2SO_4$, 20 m*M* $MgCl_2$, 0.5 m*M* spermine	Birefringent microcrystals and spherulites§

* Method 1: protein crystallization sparse matrix (Jancarik & Kim, 1991). Method 2: RNA crystallization sparse matrix (Doudna *et al.*, 1993).

† The RNA initial concentration in the drop was 2.5 mg ml^{-1}.

‡ Microcrystals appeared after 0.6–1.2 *M* $(NH_4)_2SO_4$ was added to the well, causing a further increase in concentration of all the components in the drop.

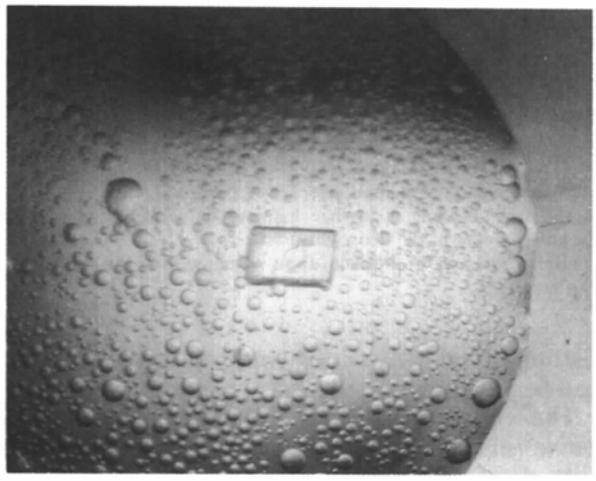
§ Spherulites were obtained upon refinement of the crystallization conditions.

The RNA content in the crystals was confirmed by native gel electrophoresis (Fig. 4). Two RNA bands were detected on 20% native polyacrylamide gels (Pharmacia Phast system) for a representative crystal dissolved in H_2O (lane 2) as well as for control samples of RNA solutions stored at 295 K for one week (lanes 1 and 3). The bands were tentatively assigned to monomeric (band *a*) and dimeric (band *b*, with lower mobility) RNA. The assignment was based on the results of storage, thermal denaturation and dimerization experiments (data not shown) using native gel electrophoresis. In our storage experiments, we observed that freshly refolded RNA solutions (see *Experimental*) when stored at room temperature for several days exhibited a slow increase of the 'dimeric' form compared to the 'monomeric' form. Thermal denaturation experiments showed that when these RNA solutions were heated to 353 K for 2 min, the 'dimeric' RNA band disappeared from the gels. Heating presumably caused the RNA to unfold and reversed the 'dimerization' process. Finally, dimerization experiments showed that certain conditions known to promote the dimerization of the HIV-1 RNA (Marquet *et al.*, 1991) (NaCl concentrations in excess of 300 m*M*, solutions of RNA more concentrated than 20 mg ml^{-1} and spermine at 1–5 m*M*) also favored the rapid appearance of 'dimeric' RNA from freshly unfolded RNA solutions. It is worth noting that a stable RNA previously described as a hairpin loop in solution (Varani, Cheong & Tinoco, 1991) proved to

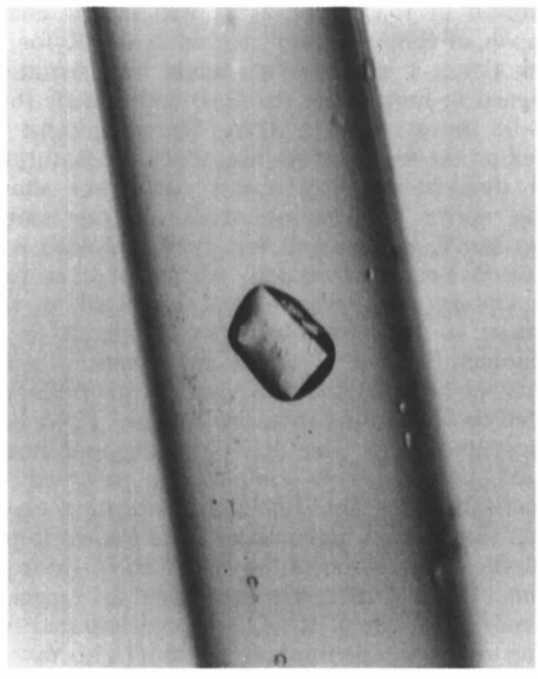
be a dodecamer duplex in the crystal structure (Holbrook *et al.*, 1991). Our RNA 'dimer' may also form an RNA double helix.

Crystallization of a complex between a 14*-residue Tat peptide and a TAR RNA fragment*

Our own adaptation (Table 1) of the incomplete factorial method of Carter & Carter (1979) (3 μl hanging drop containing one half complex and one half well solution, 1 ml of well solution, 5 mg ml^{-1} RNA, and a 1:1 molar ratio of peptide to RNA) has resulted in crystals for the complex between the TAR RNA fragment and a 14-mer peptide (Table 1, condition 65). Unfortunately, the complex between the RNA and the 9-mer peptide produced only microcrystals. The TAR RNA fragment was first incubated (see *Experimental*) in 10 m*M* K phosphate, pH 7.0 and 100 m*M* NaCl, then mixed with an equivalent volume of Tat peptide dissolved in water. A 1:1 molar ratio of RNA to peptide was used. The RNA–peptide mixture was incubated at room temperature for 30 min prior to crystallization. Strongly birefringent crystals (200 × 150 × 70 μm, Fig. 3*b*) were obtained at 295 K by the hanging-drop vapor-diffusion method. The drop (3 μl) contained an initial RNA concentration of 2 mg ml^{-1} and half of the precipitant concentration. The drop was equili-



(*a*)



(*b*)

Fig. 3. (*a*) Single crystal of the TAR RNA fragment (200 × 150 × 30 μm). (*b*) Single crystal of the peptide–RNA complex (200 × 150 × 70 μm) mounted in a capillary.

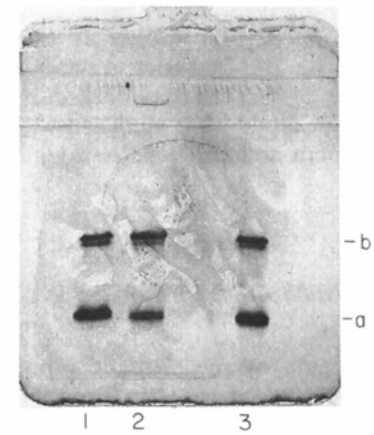


Fig. 4. Native gel electrophoresis on 20% polyacrylamide of an RNA crystal. The gel was silver-stained. Lanes 1 and 3: RNA controls stored at 295 K for several days. Lane 2: redissolved RNA crystal.

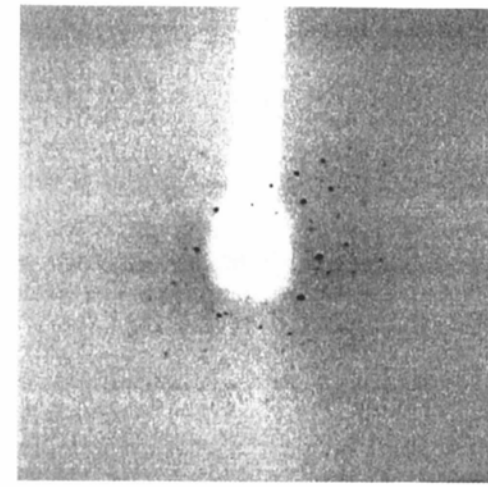


Fig. 5. 3° oscillation image of the diffraction pattern of a peptide–RNA complex for 30 min exposure.

brated against a 1 ml well solution of 50 m*M* Na cacodylate, pH 6, 50 m*M* LiCl, and 20%(*w*/*v*) PEG 3350. The crystals diffracted only to 10–15 Å (Fig. 5). We were not able to determine the space group with confidence but 3° oscillation images processed by the Molecular Structure Corporation software resulted in the following cell dimensions for triclinic crystals: $a = 60$, $b = 102$, $c = 60$ Å, $\alpha = 106.6$, $\beta = 97.9$ and $\gamma = 82°$.

The presence of RNA in a crystal of the complex was confirmed on a 20% native polyacrylamide gel (Pharmacia Phast system) shown in Fig. 6(*a*), lane 4. Band *b* with retarded mobility represented the RNA–peptide complex. The identity of this band was confirmed by its presence in RNA solutions containing both unsaturating (Fig. 6*a*, lane 2) and saturating (Fig. 6*a*, lane 3) levels of peptide. Although we were not able to calibrate our gels with oligoribonucleotides of a suitable size, other RNA bands were detected in lane 4 and deserve some discussion. Band *a* was assigned to free RNA as it was the major species detected in the RNA control (Fig. 6*a*, lane 1). Free RNA could result from the loss of peptide during extensive washing of the crystal. Band *c* seems to correspond to the RNA dimer previously described which is also visible as a faint band in the RNA control (lane 1). Band *d* seems to have the same relationship to band *c* that band *b* has with band *a* and could correspond to a peptide-bound RNA dimer. Finally, bands *e* and *f* could be the result of cooperative binding of the peptide to the RNA dimer. Further studies of these multiple complexes would require much larger crystals.

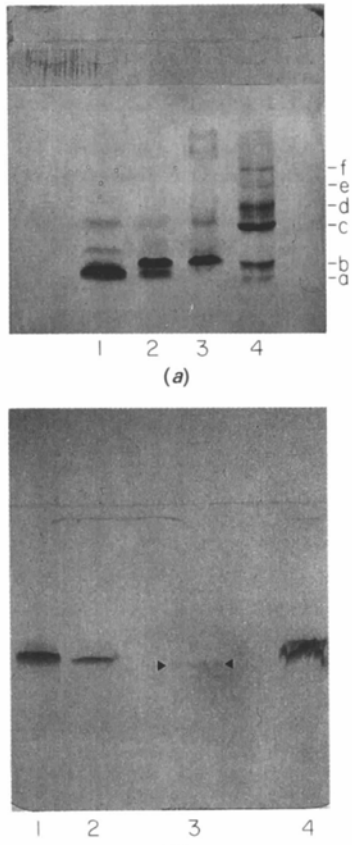


Fig. 6. (*a*) Native gel electrophoresis (with silver stain) on 20% polyacrylamide of a crystal of the peptide–RNA complex. Lane 1: RNA control, freshly unfolded by heating and quick cooling. Lane 2: solution of RNA at non-saturating peptide concentration. Lane 3: solution of RNA at saturating peptide concentration. Lane 4: redissolved crystal of the complex. (*b*) SDS–PAGE (with Coomassie stain) on a high-density gel of a crystal of the peptide–RNA complex. Lanes 1 and 2: 1 and 0.1 μg of peptide standards, respectively. Lane 3: dissolved crystal of the peptide–RNA complex (the peptide band is bracketed between two arrows). Lane 4: peptide–RNA complex in solution in a 1:1 molar ratio containing 1 μg of peptide.

The presence of peptide in a crystal of the complex was confirmed on a high-density SDS polyacrylamide gel (Pharmacia Phast system) shown in Fig. 6(*b*), lane 3. Control samples consisted of solutions containing 1 and 0.1 μg of peptide (lanes 1 and 2) and an RNA solution containing 1 molar eqivalent of peptide (lane 4). Although the peptide band in the crystal of the complex (lane 3) was detectable, it was too faint for an accurate estimate of the peptide concentration.

Concluding remarks

Single crystals of a TAR RNA fragment and of a complex between a 14-mer Tat peptide and the RNA were obtained. Although the crystals are not yet of high enough quality for structural studies by single-crystal X-ray diffraction, we have shown that crystallization of these species is possible. Better crystals might be obtained by further purification of the RNA (by HPLC anion exchange chromatography, for instance). A more homogeneous RNA preparation free of shorter transcripts may be obtained by this method. Use of longer Tat peptides might also improve crystals. Weeks & Crothers (1991) found that two longer Tat peptides which were a 24-mer peptide (residues 49–72) and a 38-mer peptide (residues 49–86) formed 50-fold more stable complexes with a TAR RNA fragment than a 14-mer peptide (spanning residues 48–61). Finally, purification of the TAR–peptide complexes on native gels prior to crystallization could improve homogeneity and result in better crystals.

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