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RNA – Synthesis, Purification and Crystallization

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Abstract

Protocols for the routine chemical synthesis and purification of milligram quantities of RNA and DNA–RNA chimeras meeting the demands of X-ray crystallography are described. An efficient screening protocol to test the crystallizability of the molecules and the optimization of the crystallization conditions are presented, so as to allow reproduction by others. Essentially the same crystallization conditions as for DNA oligomers can be employed for RNA crystallization. Specific examples involving alternating octamers, G/C-rich decamers, sequences with overhangs, and drug complexes of chimeras are discussed. Success of the methods is attested by the crystals obtained which diffract to high resolution.

1. Introduction

The availability of quick and efficient synthetic protocols and the development of reliable crystallization conditions, has established the crystallography of DNA as a tremendously successful technique since the early 1980's, and led to a revolution in DNA structure determination. RNA crystallography has so far lagged behind because for a long time its chemical synthesis was not routinely implemented on nucleic acid synthesizers (Wahl & Sundaralingam, 1995). In recent years we have seen a considerable improvement in the efficiency of RNA chemical synthesis and purification, which now almost equals that of DNA, albeit at a still tenfold greater cost. RNA sequences up to about 80 nucleotides can now be synthesized and purified (Gasparutto *et al.*, 1992; Green, Szostak, Benner, Rich & Usman, 1991; Perreault *et al.*, 1989; Scaringe, Francklyn & Usman, 1990; Usman & Cedergren, 1992; Usman, Ogilvie, Jiang & Cedergren, 1987; Whoriskey, Usman & Szostak, 1995). Although this material shows biological activity, RNA used for crystallizations is usually derived from *in vitro* transcription systems (Holbrook, Cheong, Tinoco & Kim, 1991; Pley, Flaherty & McKay, 1994). Chemically synthesized RNA was used in the determination of the crystal structure of a hammerhead ribozyme, and the great ease of obtaining heavy-atom derivatives using halogenated phosphoramidites was demonstrated (Scott *et al.*, 1995; Scott, Finch & Klug, 1995). Reports on successful crystallizations (Baeyens, Jancarik & Holbrook, 1994; Doudna *et al.*, 1993; Kim *et al.*, 1994; Pley, Lindes, DeLuca-Flaherty & McKay, 1993; Usman, Egli & Rich, 1992) and structure determinations of RNA (Baeyens, De Bondt & Holbrook, 1995; Betzel *et al.*, 1994; Cruse *et al.*, 1994; Dock-Bregeon *et al.*, 1988, 1989; Holbrook *et al.*, 1991; Leonard *et al.*, 1994; Pley *et al.*, 1994; Portmann, Usman & Egli, 1995; Schindelin *et al.*, 1995; Scott, Finch & Klug, 1995; Wahl, Ban, Sekharudu, Ramakrishnan & Sundaralingam, 1996; Wahl, Rao & Sundaralingam, 1996) and DNA–RNA chimeras (Ban, Ramakrishnan & Sundaralingam, 1994*a*,*b*; Chen, Ramakrishnan & Sundaralingam, 1995; Egli, Usman & Rich, 1993; Egli, Usman, Zhang & Rich, 1992; Wang *et al.*, 1982) are now appearing in increased numbers in the literature, illustrating the importance of the field. New, reliable protocols for the production and crystallization of RNA's are therefore still in need, to support structure–function investigations by NMR and X-ray crystallography. The vast majority of RNA and chimerical sequences studied are between six and 12 nucleotides in length, and it would be useful to have a purification/crystallization scheme, which circumvents the more elaborate methods used for larger

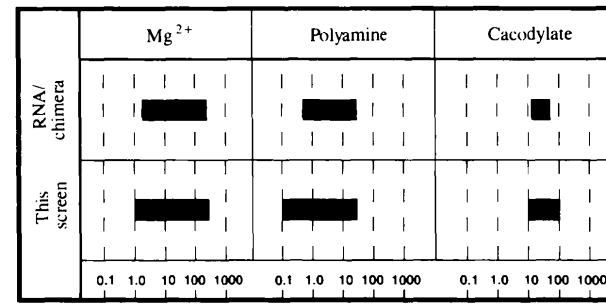

Fig. 1. Molar ranges (with respect to 1 m*M* single-stranded oligonucleotide) of magnesium, spermine and buffer concentrations employed in the crystallization of RNA and DNA–RNA chimeras. The screening protocol covers the established ranges of these components.

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molecules. Here therefore, we describe the synthesis and simple purification of short to intermediate length RNA and DNA–RNA chimeras with readily available reagents and technology, which allows us to routinely produce milligram quantities of material suitable for crystallographic work.

The chemical similarity of DNA and RNA suggests that the molecules could be crystallized under similar conditions. For DNA crystallization the variation of three main components has proven very successful: divalent metal ions, usually magnesium; polyamines, most commonly spermine; and 2-methyl-2,4-pentanediol

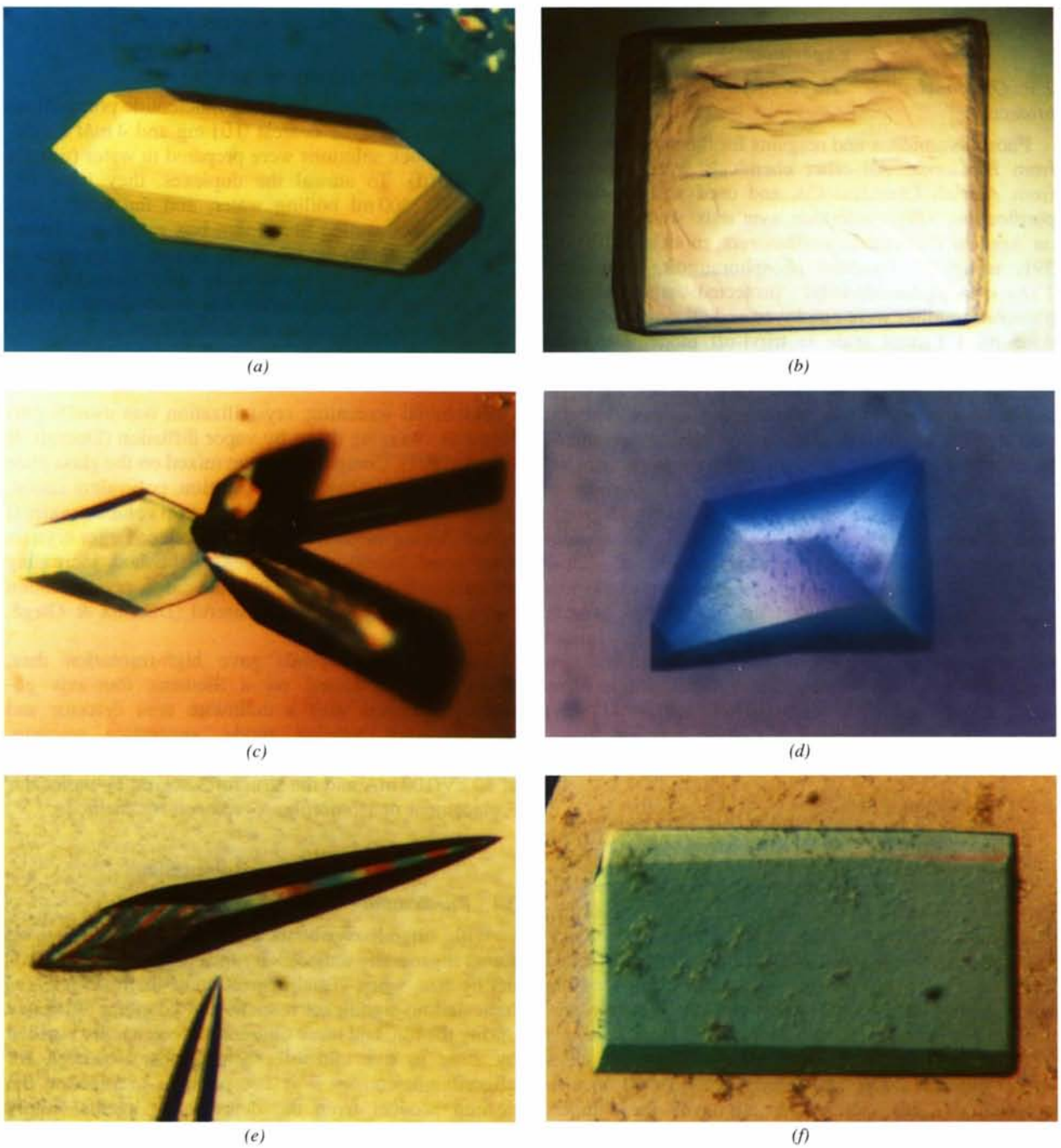

Fig. 2. Crystals grown by the present methodology. (*a*) r(GUAUAUA)d(C), rhombohedral; (*b*) r(GUAUAUA)d(C), orthorhombic; (*c*) r(GUGUACA)d(C), rhombohedral; (*d*) r(C)d(CGGCGCCG)r(G), orthorhombic; (*e*) d(I)r(C)d(ICICIC).DMA, tetragonal; (*f*) d(C)r(GUAUAUA)d(CG), monoclinic.

(MPD) as the precipitant. The pH of the solution is usually maintained between 6.0 and 7.0 by cacodylate buffer. Herein we demonstrate that variations of the same factors, together with a small set of other chemicals [calcium ions, 2-propanol, polyethylene glycol (PEG) 400, and cobalt hexammine], constitute an efficient screen for the crystallization of RNA and chimerical oligonucleotides.

2. Experimental

2.1. *Oligonucleotide chemical synthesis and deprotection*

Phosphoramidites and reagents for the syntheses were from BioGenex. All other chemicals were purchased from Aldrich Chemical Co. and used without further purification. Oligonucleotide synthesis was performed on Applied Biosystems synthesizers, models 381A and 391, using the standard phosphoramidite chemistry. 2′*O-tert*-butyl-dimethylsilyl protected ribonucleotide phosphoramidites were employed and all syntheses were done on a 1 μmol scale in trityl-off mode. For short oligomers visual monitoring of the trityl releases shows whether a size separation is necessary (see below). DNA and RNA syntheses were implemented on two different synthesizers, so chimerical molecules could be produced by changing machines at the appropriate steps.

The oligonucleotides, which were still completely protected with the exception of the 5′-hydroxyl group, were cleaved from the column with ammonium hydroxide:ethanol solution and incubated at 328 K overnight. The solution was then evaporated to dryness in a lyophilizer. To deprotect the 2′-hydroxyl groups, 100 μl tetrabutylammonium fluoride (TBAF) per 1 μmol O2′ were added. The sample slowly dissolved and was left overnight at room temperature. After addition of an equal amount of 0.1 *M* triethylamine acetate (TEAA) the mixture was evaporated in a lyophilizer and subsequently precipitated with ammonium acetate/ethanol.

2.2. *Ion-exchange FPLC*

Chromatographic separations were carried out on a Pharmacia FPLC system employing a SOURCE™15Q column (6 mm internal dimensions, 1.5 ml gel bed) for ion exchange and a C-8 column for reverse-phase runs. The sample was dissolved in 400 μl buffer *A* (0.05 *M* ammonium actetate, pH 7.0, 20% acetonitrile) and loaded onto the column at 2 ml min^{-1} flow rate. After washing with 5 ml buffer *A*, samples were eluted in a 60 min gradient to 100% buffer *B* (0.05 *M* ammonium actetate, pH 7.0, 1 *M* LiCl, 20% acetonitrile). For oligonucleotides ≤ ten bases the whole synthesis was loaded at once in a preparative run. For longer oligomers the samples were halved. The collected fraction was evaporated to dryness and ethanol precipitated several times. LiCl is not precipitated with ethanol and the sample is therefore desalted during these precipitations.

2.3. *Dialysis*

For purification by dialysis the sample was deprotected as described and dissolved in water. The solution was placed in a glass vial, capped with a 500 Da cutoff cellulose membrane, immersed in a 2 l water bath, and slowly stirred. The water reservoir was exchanged three times over the period of 3 d. The lyophilized sample was then evaporated to dryness and ethanol precipitated until a white fluffy appearance was obtained.

2.4. *Preparation for crystallization trials*

The purified, lyophilized oligonucleotide preparations were weighed to the nearest 0.01 mg and 4 m*M* single-stranded stock solutions were prepared in water (usually 100–200 μl). To anneal the duplexes, they were immersed in 500 ml boiling water, and the beaker was left in a thick-walled Styrofoam box overnight. Sometimes the assembled trials were heated in an oven to 323–343 K at which temperature they were kept for several hours. The temperature was then reduced to room temperature over a period of several days.

2.5. *Crystallization methods*

For initial screening, crystallization was usually performed in hanging drops by vapor diffusion (Ducruix & Giegé, 1992). Components were mixed on the glass plate in the order of buffer, divalent cation, polyvalent cation, precipitant, nucleic acid. For small drop volumes water is added. Sitting drops were used to produce larger crystals once good conditions had been established, increasing the recipes five to 20-fold. In some cases, crystallization by microdialysis has proved useful (Ducruix & Giegé, 1992).

Many of the crystals gave high-resolution data, which were collected on a Siemens four-axis goniostat equipped with a multiwire area detector and a Macscience rotation anode, generating graphite-monochromated Cu $K\alpha$ radiation (λ = 1.5418 Å) usually at 40 kV/100 mA and the structures solved by molecular replacement or isomorphous replacement methods.

3. Results and discussion

3.1. *Purification*

With oligodeoxyribonucleotides of ≤ 12 bases we found it generally unnecessary to separate the final product by size, when visual inspection of the trityl releases indicated no significant reduction in coupling efficiency during the run, and these oligomers are generally purified by three to five ethanol precipitations. However, for oligoribonucleotides it is not possible to separate the desired product from the deprotecting agents simply through ethanol precipitations. A single ion-exchange FPLC run was usually found sufficient to avoid salt crystals. Ion-exchange FPLC was preferred over reverse-phase FPLC because of the higher loading capacity of

Table 1. *Percent usage of crystallization components of nucleic acids*

This table was compiled from information in the Nucleic Acid Database.

	Polyamine (%)	Mg^{2+} (%)	Co/Rh/Ir-Hexammine (%)	Cacodylate (%)	MPD in drop (%)	MPD in reservoir (%)	Isopropanol (%)
A-DNA	76	72	8	82	64	88	5
B-DNA	71	88	—	51	83	96	—
Z-DNA	59	81	5	66	68	79	26
RNA/chimera	50	60	10	80	40	80	—
This screen	71	75	13	96	71	79	17

the column used and to circumvent trityl-on chromatography. Instead of the linear gradients, step gradients may be used for short RNA oligomers (≤ ten bases), which reduces sample loss. For oligonucleotides longer than 20 bases, usually stronger denaturing conditions are required to size-separate them in FPLC, *e.g.* using heated columns (Murray, Collier & Arnold, 1994). Alternatively the samples may be gel-purified (Pley *et al.*, 1993; Price, Ito, Oubridge, Avis & Nagai, 1995). Scott *et al.* (Scott, Finch, Grenfell, 1995) reported improved yields for chemically synthesized hammerhead ribozyme sequences when they employed a deoxy-solid support and we observed a similar phenomenon. However, in the present case one reason may be, that oligomers which contain a few deoxy-nucleotides were more easily separated on the FPLC than the all-ribo analogs.

One-step dialysis purification was explored when trityl releases indicated efficient coupling. The products from the dialysis could be crystallized equally successfully as those obtained from FPLC purification. Both purification methods usually yielded 0.4–0.7 μmol final product (40–70% yield). They can, therefore, produce milligram quantities of material suitable for crystallographic purposes.

3.2. *Crystallization*

3.2.1. *Buffers and pH.* DNA oligomer crystallizations are usually not very dependent on the pH, because of the absence of groups with pK_a values near neutral pH (Ducruix & Giegé, 1992). Acidic pH's may be attempted when protonated structures are to be stabilized (Rubin, Brennan & Sundaralingam, 1971; Seeman, Sussman, Berman & Kim, 1971). The DNA–RNA chimeras and RNA oligomers which were screened crystallized over the pH range 6.0–8.0. Fine adjustment of the pH was sometimes useful to slow crystal growth, with crystals appearing faster at lower pH. As evident from Table 1, the most commonly employed buffer for DNA crystallizations is cacodylate. It is also popular in the few RNA oligomer crystallizations reported and was used in most trials in our screening at pH 6.0, 6.5, and 7.0. Tris buffer is less frequently employed and only one trial in the screen uses Tris–HCl at pH 8.0. It should be noted that some ions in the screen (see below) have acidic or basic character and possibly some buffering capacity. For this work, the pH has generally not been tested after the preparation of the drops.

3.2.2. *Cations.* Although cations are not always located in the electron-density map of a nucleic acid crystal structure they are usually indispensable for production of high-quality crystals. Presumably they are disordered, occupying channels in the crystal, and allow the negatively charged nucleic acid molecules to approach each other. Monovalent ions like sodium, potassium, or ammonium may be used to solubilize some oligonucleotides, or in higher concentrations as precipitants. They were not included as a variant in the initial screening but are sometimes useful in improving crystal quality.

Magnesium ions are most prominent among the divalent metal ions used in nucleic acid crystallography with calcium ions in the second place (Table 1). In this work, magnesium was used in chloride or acetate form and the nature of the anion was generally not found to be important for the crystallizations. It is worthwhile to test the effect of other divalent metal ions when magnesium or calcium do not yield suitable crystals. Mg^{2+} was screened across the concentration range employed in other RNA crystallizations (Fig. 1).

Spermine was the polyamine used exclusively for initial screening purposes because it is the most popular polyamine used in nucleic acid crystallography (Table 1). Fig. 1 shows the ranges of spermine concentrations (relative to the single-stranded oligomer) which have been employed for RNA and DNA–RNA chimera crystallizations in the past. Spermine concentrations covering these values were included in the screening.

We found that cobalt hexammine can be very successful in the crystallization of RNA and chimeras. This finding is in agreement with the observation that this complex ion promotes the DNA A-form (Bingman, Jain, Zon & Sundaralingam, 1992; Bingman, Zon & Sundaralingam, 1992; Ramakrishnan & Sundaralingam, 1993). Several cobalt hexammine conditions were incorporated in the screen. One drawback is that it tends to form salt crystals at higher concentrations. In one reported instance, rhodium and iridium hexammines were used to crystallize an RNA duplex (Cruse *et al.*, 1994). All these metal subgroup VIIIB hexammines may therefore be useful components in crystallizations of RNA's and chimeras. Although we have not located any cobalt

hexammine in the crystal structures solved to date, it may bind site-specifically in some structures. Crystals may then exhibit a slight orange color. When cobalt hexammine is present in the structure, anomalous data can also be collected. In general, it was found that crystal growth was often critically dependent on the concentration of the polyvalent cations, although other factors can often be varied by an order of magnitude.

We examined several RNA and DNA–RNA chimerical molecules for their capacity to bind cationic minor-groove drugs. Drugs can be easily incorporated into the screen by adding a constant amount to each drop (usually 1–10 m*M*). Crystals which contain drug molecules sometimes can be identified by their color. Because minor-groove binding drugs are usually cations they may be helpful ingredients even for molecules which do not bind them specifically.

3.2.3. *Precipitants*. The precipitants used are 2-propanol, polyethylene glycol (PEG) 400, and 2-methyl-2,4-pentanediol (MPD). MPD is by far the most successful precipitant used for DNA oligonucleotide crystal growth (Table 1). However, changing the precipitant is certainly an important consideration when conditions are expanded (see below). For RNA crystallography low molecular weight PEG's have received some attention (Baeyens *et al.*, 1994) and also ammonium sulfate is sometimes a good choice.

While MPD and PEG were often not only contained in the reservoir but also added to the drop, 2-propanol was only added to the reservoir because it will quickly diffuse into the drop through the gas phase. Reservoirs consisted of either 1 ml 60% MPD or PEG, or 5–10 µl 10% 2-propanol. In order to slow down the vapor diffusion of 2-propanol into the drop, the reservoir was kept small and was replenished when it appeared to have dried out. MPD in the drop sometimes was the origin of phase separations which could usually be avoided by omitting it or changing the temperature. The reservoirs generally contained only the precipitants at higher concentrations because inclusion of all other drop components was found to be unnecessary.

3.2.4. *Temperature*. Crystallizations were first attempted at higher temperatures (291–295 K). The culture plates can then be either moved to lower temperatures (277–280 K) or a separate trial can be started at lower temperatures when no crystals have appeared after several weeks. For some oligomers it was found that temperatures of > 303 K produced crystals. Similar results have been reported for a few RNA molecules (Dock-Bregeon *et al.*, 1989; Schindelin *et al.*, 1995). The higher temperature requirement may reflect the formation of an otherwise thermodynamically unfavorable, distorted structure. We observed that for some structures crystals appeared only after annealing them again in the tissue-culture plates.

3.2.5. *Screening and optimization*. The 24-trial screen listed in Table 2 fits exactly on a 24-well tissue-culture plate and can be set up in about 45 min. The conditions are derived from DNA oligomer crystallization recipes which were found exceptionally useful for RNA and DNA–RNA chimera crystallizations. When the initial screening does not produce any crystalline material, the number of the screen conditions can be easily augmented by changing the precipitant. MPD can be replaced by 2-propanol, ammonium sulfate, or PEG of a variety of molecular weight ranges, keeping the other factors the same. Similarly, the divalent metal ions can be exchanged for others, and instead of spermine, spermidine and putrescine can be tried.

Crystals which exhibited the best shapes, not necessarily the biggest crystals, were selected for further screening, and shape was the major criterion during the following optimization steps. It was generally first tried to vary the concentrations of individual components, to omit individual components, to leave precipitants out of the drop, to change the temperature, and to change buffer pH. When unsuccessful, it was attempted to change the chemical nature of the components. The nature of the precipitant was also varied. When suitable conditions were established, the precipitant concentration in the reservoirs was reduced to 10–40%. However, it was found that the main factor in the production of large good quality crystals was not time, but the correct mixture of components. With the right ingredients established, the crystallizations were very rugged with respect to total drop volume and reservoir concentration.

3.3 *Specific examples*

A sample of structures crystallized according to the techniques outlined is listed in Table 3 and representative crystals are shown in Fig. 2. Among these are four groups which will be discussed in some detail: alternating octamer RNA's with 3′-terminal deoxycytidine residues; G/C-rich decamers; RNA's with overhangs; and alternating octameric chimeras with drugs. The longest sequence which was synthesized and gave well diffracting crystals was the 15-mer, r(CGCGCGUUUCGCGCG). Longer oligomers were prepared but so far yielded only poorly diffracting crystals (Table 3).

3.3.1. *Alternating octamers*. Cobalt hexammine in low concentrations was the critical factor in crystallizing the molecules. Sharp-edged large crystals grew under conditions of the screen and did not need to be improved further (Figs. 2*a*–2*c*). Diffraction power was very sequence dependent. The molecules crystallized in space group *R*3 with one duplex per asymmetric unit (Wahl, Ban *et al.*, 1996). However, when MPD was omitted from the drop, the crystal system changed to orthorhombic with an estimated two duplexes per asymmetric unit. The orthorhombic crystals were more fragile and cracked easily when mother liquor was

Table 2. *Screening conditions*

Condition	Divalent cation*	Polyamine*	Cobalt hexammine*	Buffer*†	Precipitant in drop (10%)	Reservoir
1	$MgCl_2$ 1			Na caco. (pH 7.0) 20		10% 2-Propanol
2	$MgCl_2$ 1			Na caco. (pH 7.0) 30	MPD	60% MPD
3	$MgCl_2$ 100			Na caco. (pH 6.5) 30	MPD	60% MPD
4	$MgCl_2$			Tris-HCl (pH 8.0) 10	MPD	60% MPD
5	$MgCl_2$ 1	Spermine/$(HCl)_4$ 1		Na caco. (pH 7.0) 100		10% 2-Propanol
6	$MgCl_2$ 1	Spermine/$(HCl)_4$ 10		Na caco. (pH 7.0) 30		10% 2-Propanol
7	$MgCl_2$ 10	Spermine/$(HCl)_4$ 1		Na caco. (pH 7.0) 30		10% 2-Propanol
8	$MgCl_2$ 10	Spermine/$(HCl)_4$ 5		Na caco. (pH 7.0) 20		60% MPD
9	$MgCl_2$ 1	Spermine/$(HCl)_4$ 1		Na caco. (pH 6.5) 10	MPD	60% MPD
10	$MgCl_2$ 5	Spermine/$(HCl)_4$ 0.1		Na caco. (pH 7.0) 40	MPD	60% MPD
11	$MgCl_2$ 5	Spermine/$(HCl)_4$ 50		Na caco. (pH 7.0) 20	MPD	60% MPD
12	$MgCl_2$ 10	Spermine/$(HCl)_4$ 10		Na caco. (pH 6.0) 20	MPD	60% MPD
13	$MgCl_2$ 100	Spermine/$(HCl)_4$ 1		Na caco. (pH 7.0) 40	MPD	60% MPD
14	$MgCl_2$ 30	Spermine/$(HCl)_4$ 1		Na caco. (pH 7.0) 10	PEG 400	60% PEG 400
15	$Mg(OAc)_2$ 250			Na caco. (pH 7.0) 10	MPD	60% MPD
16	$Mg(OAc)_2$ 10	Spermine/$(HCl)_4$ 1		Na caco. (pH 7.0) 20	MPD	60% MPD
17	$Mg(OAc)_2$ 50	Spermine/$(HCl)_4$ 1		Na caco. (pH 7.0) 10	MPD	60% MPD
18		Spermine/$(HCl)_4$ 5		Na caco. (pH 7.0) 40	MPD	60% MPD
19	$CaCl_2$ 5	Spermine/$(HCl)_4$ 1		Na caco. (pH 7.0) 30	MPD	60% MPD
20	$CaCl_2$ 20	Spermine/$(HCl)_4$ 4		Na caco. (pH 7.0) 10	MPD	60% MPD
21	$CaCl_2$ 250	Spermine/$(HCl)_4$ 1		Na caco. (pH 7.0) 10	MPD	60% MPD
22			$Co(NH_3)_6Cl_3$ 0.5	Na caco. (pH 6.0) 40		60% MPD
23		Spermine/$(HCl)_4$ 2	$Co(NH_3)_6Cl_3$ 4	Na caco. (pH 6.5) 10		60% MPD
24	$MgCl_3$ 10 $CaCl_2$ 10		$Co(NH_3)_6Cl_3$ 10	Na caco. (pH 7.0) 15		60% MPD

*Numbers indicate molar ratios. †Na caco. = sodium cacodylate.

Table 3. *Examples of RNA and chimera crystals and the data resolution*

Sequence†	Crystallization condition‡	Crystal size (mm)	Resolution (Å)	Space group
uucgcg	7	1.0 × 0.2 × 0.2	1.4	$C222_1$
guauauaC	22	0.6 × 0.2 × 0.2	2.2	$R3$
guguacaC	3	0.3 × 0.2 × 0.2	2.5	$R3$
guauguaC	22	0.3 × 0.2 × 0.2	1.4	$R3$
guacauaC	24	0.7 × 0.4 × 0.2	2.5	$I222$
icicicic/DMA§	3/DMA	0.3 × 0.15 × 0.15	2.6	$P222$
IcICICIC/DMA	16/DMA	0.5 × 0.08 × 0.08	1.7	$P4_122$
IcIcICIC/DMA	16/DMA	0.2 × 0.08 × 0.08	1.6	$P4_122$
IcAuATIC/DMA	13/DMA	0.3 × 0.1 × 0.1	1.9	$P4_122$
GuAuATAC/DMA	22/DMA	0.5 × 0.2 × 0.2	1.5	$C2$
CCGGCgCCGG	22	0.7 × 0.3 × 0.15	1.9	$P2_12_12_1$
cCGGCGCCGg	22	0.2 × 0.2 × 0.2	1.9	$P2_12_12_1$
CCGGcGCCGG	22	0.3 × 0.2 × 0.2	1.8	$P2_12_12_1$
CCGGcgCCGG	22	0.7 × 0.3 × 0.1	1.9	$P2_12_12_1$
ccggcgccgG	22	0.3 × 0.2 × 0.2	1.6	$R32$
ccggcgccgg	22	0.3 × 0.3 × 0.3	2.2	$R32$
cgugcgcccG	11	0.3 × 0.3 × 0.2	1.6	$R32$
cgcgcguuucgcgcg	11	0.4 × 0.3 × 0.2	2.8	$P2$
(COL/COL*)¶	10	0.2 × 0.2 × 0.2	14.7	Not determined

†Lower-case characters = RNA, upper-case characters = DNA. ‡Crystallization conditions are the best conditions identified with the screen and were sometimes slightly varied to achieve better crystals. §DMA = Distamycin A; DMA concentrations were 1–2 m*M* in each case. ¶(COL/COL*) is a system of kissing hairpins with 5′-UU-overhangs: r(UUGUGCGAACCACGCAC)/r(UUGACGAGUGGUUUCGUC).

removed. For RNA a change in the divalent/polyvalent cation concentrations often effects a change in the space group. However, change in space group upon omission of a precipitant has not been reported yet. We had difficulties growing crystals of the all-ribo variants of the octamers, although we see no obvious reason from the crystal structure.

3.3.2. *G/C-rich decamers.* The chimerical decamers d(CCGGC)r(G)d(CCGG) (1); r(C)d(CGGCGCCG)r(G) (2); d(CCGG)r(C)d(GCCGG) (3) and d(CCGG)r(CG)-d(CCGG) (4), crystallized in the A-form and were derived from the sequence d(CCGGCGCCGG) which crystallized in the B-form (Heinemann, Alings, & Bansal, 1992). They were all purified by dialysis. All

sequences crystallized as bent A-form helices (Ban *et al.*, 1994*a*,*b*) (Fig. 2*d*). No magnesium or other divalent metal ions were employed but the spermine concentration was very critical in these cases. While chimeras 1 and 2 yielded high-quality crystals under low spermine concentrations (2–2.5 m*M*), chimeras 3 and 4 required higher spermine (5–10 m*M*). All crystals diffracted to high resolution but chimera 2 exhibited considerable mosaic spread. The quality of the chimera 2 data was vastly improved after soaking the crystals overnight in 75% MPD. This procedure is reminiscent of the soaking of crystals in cryoprotectant for investigation at low temperatures. Interestingly, the all-RNA analog, r(CCGGCGCCGG), and the chimera, r(CCGGCGCCG)d(G), crystallized under similar conditions as chimeras 3 and 4, but in a different space group (*R*32). Possibly the presence of the 2′-hydroxyl group at the 3′ end of the all-RNA molecule denies the typical A-DNA packing (end-base pair, minor-groove stacking), and a high ribose content may favor coaxial stacking of the duplexes by providing additional 'handles' for lateral fixation. In agreement with the latter suggestion, the quite different sequence, r(CGUGCGCCC)d(G), crystallized isomorphously in R32. However, crystallization conditions had to be changed; magnesium was now found to be a critical component and the spermine concentration had to be increased to 35–50 m*M*.

3.3.3. *Sequences with overhangs.* In various examples it has proven useful to include overhanging nucleotides in the molecules to be crystallized. These overhangs can effectively mediate crystal packing, either through base-pairing (Cruse *et al.*, 1994) or through the interaction of the overhangs with the grooves of a symmetry-related duplex (van Meervelt *et al.*, 1995). Both 3′- and 5′-overhangs may be useful. When complementary overhangs are used, which are intended to associate through base pairing, the length of the overhang could be adjusted so that stacked duplexes would be in approximate register with the repeat of crystallographic screw axes. The sequence r(UUCGCG), *e.g.*, forms infinite double helices in the crystal, in which the CGCG portions are Watson–Crick base paired and the 5′-terminal UU overhangs form self pairs (Wahl, Rao *et al.*, 1996). The crystals form under a variety of conditions and the helices are aligned along a 2_1-screw axis. Overhanging nucleotides may not only be useful for providing an effective means of packing in one dimension, but they also may alleviate the end effects on the duplexes by extending them to infinity.

3.4.4. *Drug complexes of DNA–RNA chimeras.* Minor-groove binding drugs were thought to bind only to B-DNA for a long time and not to DNA–RNA chimeras because of the tendency of the latter to adopt the A-form (Ban *et al.*, 1994*a*,*b*). But surprisingly several DNA–RNA chimeras cocrystallized with distamycin A (DMA) and the presence of the drug was found to be absolutely essential for obtaining crystals (Chen *et al.*, 1995). Unlike the DNA counterpart, $d(IC)_4$ in complex with DMA (Chen, Ramakrishnan, Rao & Sundaralingam, 1994), crystallization of the drug complexes of the chimeras, d(I)r(C)d(ICICIC) and d(I)r(C)d(I)r(C)d(ICIC), posed quite a problem. Small variations of the drug-to-oligonucleotide ratio proved to be not critical. Larger ratios tended to promote nucleation, similar to the effect of increasing polyamine. An elevation of the Mg^{2+} concentration in concert with a careful tuning of the MPD concentration in the droplet improved the size and quality of the crystals (Fig. 2*e*), while spermine was dispensable. Interestingly, the drug was also found to be critical in crystallizing an all-RNA octamer, r(IC)4, although the drug seemed not specifically complexed with the RNA in the crystals, which seemed to adopt the A-form (Pan & Sundaralingam, unpublished results). Drugs may prove to be useful in crystallizing tough RNA sequences by stabilizing the RNA or the crystal lattice specifically or nonspecifically.

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