

# Large scale chemical synthesis, purification and crystallization of RNA-DNA chimeras

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## ABSTRACT

**RNA-DNA chimeras, in which both DNA and RNA monomers are site-specifically substituted in the same strand, may be prepared only by chemical synthesis. Biochemical studies have revealed a number of surprising and subtle effects resulting from the insertion of either a ribonucleotide into a DNA strand or a deoxyribonucleotide into an RNA strand. The availability of large quantities of these chimeras allows for their crystallization and subsequent x-ray structure determination. We describe a flexible and efficient method for the large-scale preparation of these compounds, their purification, and their crystallization. The methodology is based on a combination of existing DNA phosphoramidite synthons and those recently introduced for the preparation of biochemically active RNA<sup>1</sup>. We demonstrate that these two different synthons are compatible, produce large quantities of nucleic acid needed for physical studies, and that high resolution diffraction quality crystals may be grown from these chimeras. Of the duplex chimeras synthesized and crystallized, [r(G)d(CGTATACGC)]<sub>2</sub>, [d(GCGT)r(A)d(TACGC)]<sub>2</sub> and [r(GCG)d(TATACCC) + d(GGGTATACGC)] form A-helices and d(CG)r(CG)-d(CG)]<sub>2</sub> forms a left-handed Z-helix.**

## INTRODUCTION

In addition to the well known roles of tRNA, mRNA and rRNA in cellular processes, RNA molecules also possess catalytic activity<sup>2–6</sup>. RNA-DNA chimeras are less common, the best known example being the Okazaki fragment<sup>7</sup>. In this case a chimeric RNA-DNA molecule is base-paired to an all DNA strand to form a hybrid RNA·DNA duplex contiguous to a DNA·DNA duplex. Recent studies on the mechanism of action of hammerhead ribozymes indicate that RNA-DNA chimeras, both in single-stranded and duplex regions are also catalytically active<sup>8–10</sup>. In the interaction between an aminoacyl synthetase and chemically synthesized substrates deoxyribonucleotide substitutions in the duplex RNA substrate may be tolerated by the enzyme<sup>11,12</sup>. Substrate recognition by the *Tetrahymena* Group I self-splicing has been investigated using RNA-DNA chimeras base-paired to an all RNA internal guide sequence and

indicate that specific 2'-hydroxyls in the substrate are required for binding<sup>13</sup>. The question thus arises, what is the overall structure of duplex RNA-DNA chimeras and how does their structure affect RNA-RNA and RNA-protein molecular recognition processes?

DNA duplexes, under conditions of normal humidity and salt concentration, are usually found in the B-conformation<sup>14</sup>. Under special conditions both A-type and Z-type helices may also form<sup>14</sup>. In the case of single crystal structures, all DNA helices of the A-type have either a GG sequence at both ends of the duplexes or are octamers in length. These two characteristics fulfill the crystal packing requirements for A-form DNA. On the other hand RNA duplexes always form A-type helices<sup>14</sup>. Hybrid DNA·RNA duplexes, in which one strand is all-RNA and the other all-DNA also exist in the A-conformation. In the two reported x-ray structures of RNA-DNA chimeras the self-complementary sequences [r(GCG)d(TATACGC)]<sub>2</sub><sup>15</sup> and d(CG)r(CG)d(CG)]<sub>2</sub><sup>16</sup> were found to be in the A conformation and the left-handed Z-conformation respectively. We were interested in studying the effect of more subtle single ribonucleotide substitutions in a DNA strand and in Okazaki fragments where one strand is completely DNA and the other an RNA-DNA chimera. To study these molecules a synthetic strategy was required which is both compatible with existing large scale solid phase DNA synthesis and results in chimeric products of sufficient quantity and purity to be crystallized following deprotection and purification.

The solid phase chemical synthesis of RNA, using 2'-*O*-*t*-butyldimethylsilyl (TBDMS) ether protected  $\beta$ -cyanoethyl phosphoramidites (Figure 1) has only recently become an efficacious method for the preparation of RNA. The description of the preparation of biochemically active RNA fragments<sup>1</sup> using conventional deoxyribonucleoside phosphoramidite protecting groups: DMT for the 5'-hydroxyl, Bz for adenosine and cytidine and *i*Bu for guanosine exocyclic amino groups, and  $\beta$ -cyanoethyl for phosphate protection, in conjunction with *t*-butyldimethylsilyl for 2'-hydroxyl protection has alleviated some of the problems of purity and yield in the chemical synthesis of RNA. Furthermore the use of the same protecting groups renders the DNA and RNA phosphoramidite synthons compatible from a synthesis and deprotection viewpoint. There have been three other descriptions of RNA and RNA-DNA chimera syntheses

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using methyl protected phosphoramidites and a variety of base protecting groups<sup>17,18</sup>. However the use of methyl phosphate protection can lead to base modification during synthesis and deprotection<sup>19</sup> and has been superseded by  $\beta$ -cyanoethyl protection which does not require an additional deprotection step and yields nucleic acids having higher biochemical activity<sup>20</sup>. The  $\beta$ -cyanoethyl phosphate protecting group has been used for the synthesis of chimeras with 2'-*O*-nitrophenyl protected ribonucleosides<sup>21</sup>, however the removal of the 2'-*O*-protecting group requires a photochemical cleavage step. In all of these aforementioned syntheses the synthetic nucleic acid has not been crystallized, although they have been used for biochemical and NMR studies.

In the chemical synthesis of RNA the potential exists of forming undesired 5'-2' internucleotidic linkages, resulting from the incorporation of isomerically impure ribonucleoside phosphoramidites during synthesis or loss or migration of the 2'-silyl protecting group during either synthesis or deprotection. Furthermore heterocyclic base modifications may also occur during these steps. These potential modifications have been examined by a number of physical and enzymatic methods and were not detected<sup>1</sup>. But the final test for complete chemical homogeneity would be crystallization of the product and subsequent structural determination by x-ray crystallography. Any nucleoside base modification, migration of the phosphate linkage, or other degradation of the nucleic acid would introduce inhomogeneities and thus prevent crystallization of the molecule or, if it indeed did crystallize, would appear in the 3D structure.

We demonstrate that the DNA and RNA phosphoramidite methodologies used in this work are completely compatible and produce RNA-DNA chimeras which may be crystallized. The resulting crystals are of sufficient quality to permit high resolution diffraction data to be collected. The subsequently determined crystal structures showed that the RNA-DNA chimeras are free of base modifications, 5'-2' linkages, and unremoved protecting groups.

## RESULTS AND DISCUSSION

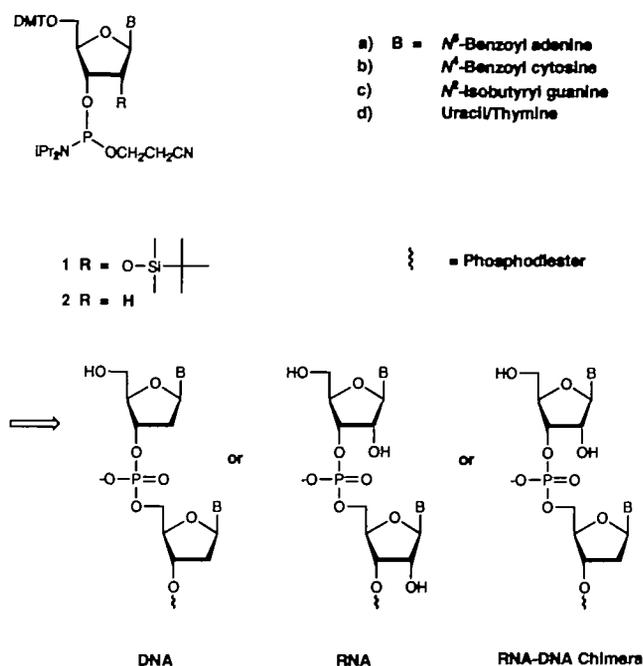
### Large scale synthesis of RNA-DNA chimeras

Sequences of the synthesized strands are shown in Table 1. Strand 3 had been previously prepared by a solution phase synthesis employing 2'-*O*-methoxytetrahydropyranyl protection and was subsequently crystallized and shown to be in the Z-conformation<sup>16</sup>. Strands 4–6 were derived from the self-complementary strand 7 with ribonucleotide substitutions. In the case of 4 and 5 it was of interest to examine the structural effect of the introduction of a single ribonucleotide into an otherwise all-DNA double-helix. The chimeric RNA-DNA strand 6 was paired with its complementary all-DNA strand to generate an Okazaki fragment<sup>22</sup>.

The 2'-*O*-TBDMS  $\beta$ -cyanoethyl ribonucleoside phosphoramidites<sup>1</sup> **1a–d** shown in Figure 1 were used in conjunction with standard DNA phosphoramidites **2a–d**. These reagents have been used successfully in numerous biochemical studies in which small-scale, 0.5–1.0  $\mu$ mol, syntheses were performed (for a review see reference 23). For the large-scale synthesis at ribonucleotide positions a 10  $\mu$ mol protocol for DNA synthesis was used with one modification, a 10 min coupling time was used for the ribonucleotides vs 1 min for the deoxyribonucleotides. The overall cycle was identical for the ancillary steps of detritylation, capping, oxidation and washing.

**Table 1.** Cell constants and space groups of chimeric RNA-DNA and DNA crystals

Sequence	Unit Cell Constants	Space Group
3 [d(CG)(CG)(CG)] <sub>2</sub>	a = 18.22Å, b = 30.82Å, c = 42.86Å	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
4 [r(G)(CGTATACGC)] <sub>2</sub>	a = 27.50Å, b = 44.23Å, c = 44.61Å	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
5 [d(GCGT)(A)d(TACGC)] <sub>2</sub>	a = 25.25Å, b = 45.73Å, c = 45.83Å	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
6 r(GCG)(TATACCC) + d(GGGTATACGC)	a = 24.03Å, b = 43.67Å, c = 48.95Å	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
7 [d(GCGTATACGC)] <sub>2</sub>	a = b = 27.3 Å, c = 132.2 Å	Hexagonal



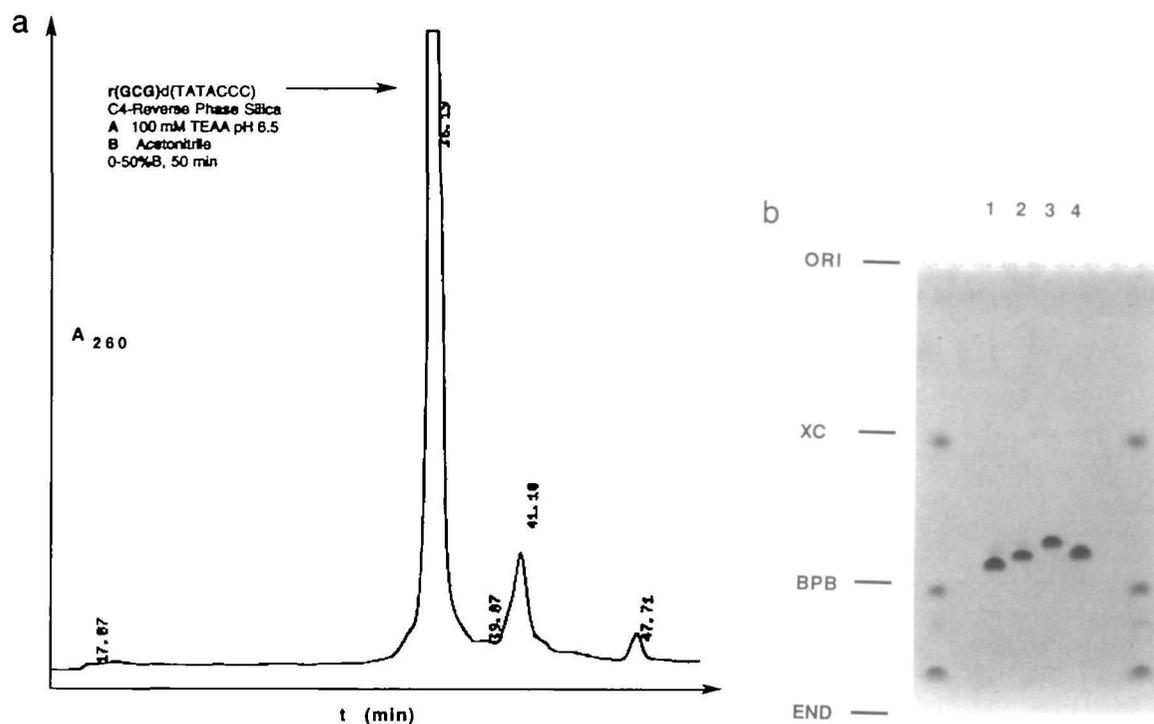
**Figure 1.** Ribo- and deoxyribonucleoside phosphoramidite synthons used for the synthesis of the chimeras.

It is important to note the compatibility of the RNA and DNA phosphoramidites. Since no change in the chemistry of synthesis is required, this method is generally available to anyone capable of carrying out DNA synthesis.

The concentration of phosphoramidites for both RNA and DNA was 100 mM. The solid phase syntheses were performed on an ABI 380B DNA synthesizer with a 10  $\mu$ mol scale using DNA-derivatized 500 Å CPG supports. A 10-fold excess of ribonucleoside phosphoramidite and ~100 fold excess of tetrazole were used. Average stepwise coupling yields, determined spectrophotometrically by A<sub>504</sub> of the released dimethoxytrityl cation, were ~98%. Other reagents and details are described in the experimental section.

### Deprotection and purification of chemically synthesized RNA-DNA chimeras

**Deprotection.** The base labile protecting groups used for the exocyclic amino and phosphate groups are the same in the DNA and RNA synthons **1,2 a–d**, thus we did not anticipate any difficulty in removing them at the end of the synthesis. The only precaution employed was the use of ethanolic ammonia vs



**Figure 2.** a) HPLC trace of crude r(GCG)d(TATACCC). b) UV shadowing photograph of an analytical 20%/7M urea PAGE of HPLC-purified RNA-DNA chimeras. 1 d(GCGT)r(A)d(TACGC), 2 r(G)d(CGTATACGC), 3 r(GCG)d(TATACCC), 4 d(CG)r(CG)d(CG). The positions of the origin (ORI), xylene cyanol FF dye (XC), bromophenol blue dye (BPB) and the end (END) are indicated.

aqueous ammonia at 55°C for 16 hr to remove the *N*-acyl protecting groups, eliminate the  $\beta$ -cyanoethyl phosphate protecting group, and cleave the chimera from the CPG support. The aqueous ammonia reagent is commonly used for DNA oligomers whereas the ethanolic one is needed for the deprotection of RNA. The use of ethanolic ammonia prevents the undesirable loss of the silyl protecting groups during this base catalyzed deprotection step<sup>1</sup>. To ensure that the use of ethanolic ammonia did not affect the deprotection of the DNA residues, samples of the all-DNA decamer 7 were treated with both reagents. HPLC analyses of the two samples were identical (data not shown) indicating that the deprotection was complete in both cases.

Although it has been shown that efficient and complete deprotection of the 2'-*O*-silyl protecting groups in all-RNA oligomers is possible using TBAF in THF, we investigated whether the RNA-DNA chimeras 3–6 could also be deprotected in a similar manner since they are comprised mainly of DNA units. Using the previously described method<sup>1</sup> of solubilizing the residue, obtained after lyophilization of the ammonia reagent, with 50:50/EtOH:H<sub>2</sub>O prior to adding the 1M TBAF/THF reagent, no solubility problems occurred.

The chimeras were treated with 50 equivalents of TBAF per TBDMS group for 24 hr. The workup of this reaction was similar to that developed for the synthesis of all-RNA sequences<sup>1</sup>. The samples of a 10  $\mu$ mol synthesis were divided in two. Each half was desalted on a Qiagen 500 anion exchange cartridge. This procedure yielded ~ 65 crude ODU per nucleotide residue and no loss of material was noted by this method.

**Purification.** The RNA-DNA chimeras were purified to homogeneity using HPLC. In our hands the use of C4-reversed phase silica gel columns has provided high quality DNA

oligomers for crystal growth. We therefore used this matrix for the purification of the chimeras with similar success. The mobile phase consisted of 100 mM TEAA pH 6.5 and the chimeras were eluted with various gradients of acetonitrile. In all cases the desired full-length product was the major peak. A typical HPLC trace is shown in Figure 2a. Following lyophilization of the mobile phase the oligomers were checked by both analytical HPLC and denaturing gel electrophoresis. Figure 2b shows a UV shadowing photograph of all four chimeric sequences purified by HPLC. The profile of the crude sequences, comprised of only a single major peak, indicates the efficacy of the synthesis and deprotection methods. The overall yield of purified chimera from  $\mu$ mol of nucleoside attached to CPG to the purified final product was ~ 50–60%. The chimeras were then used directly for crystal growth experiments.

#### Crystallization and initial crystallographic results

Crystals of [d(GCGT)r(A)d(TACGC)]<sub>2</sub>, [r(G)d(CGTATACGC)]<sub>2</sub>, and [r(GCG)d(TATACCC) + d(GGGTATACGC)] were grown at 18°C in sitting drops, using the vapor diffusion technique. The crystallization conditions in the three cases were similar (see Experimental). Photographs of crystals of the three RNA-DNA chimeras are shown in Figures 3a, b and c, respectively. For the self-complementary chimeras, optimal crystals were obtained at pH 6, whereas crystals of the Okazaki fragment were grown at pH values between 7 and 7.5. In the case of the self-complementary chimeras, moderate magnesium chloride and spermine tetrachloride concentrations were used in the crystallization dips (about 5 times higher than the concentration of single-stranded RNA-DNA chimera). However, diffraction quality crystals of the Okazaki fragment could only be grown with unusually high spermine concentrations, which

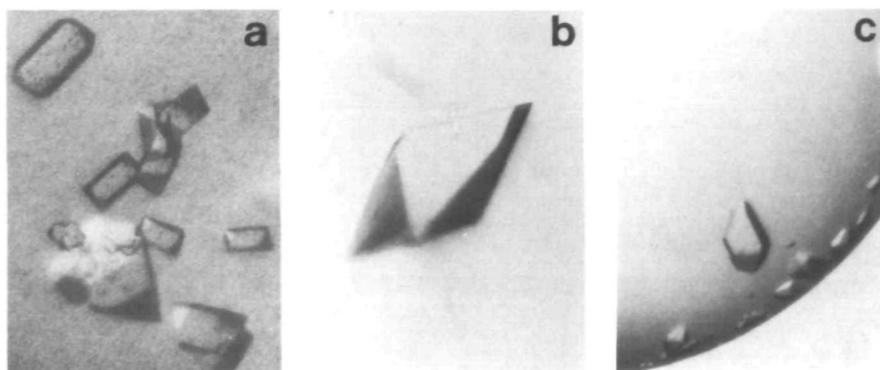


Figure 3. Photographs of RNA-DNA chimera crystals. a)  $[d(GCGT)r(A)TACGC]_2$ , b)  $[r(G)d(CGTATACGC)]_2$ , c)  $[r(GCG)d(TATACCC) + d(GGGTATACGC)]$

were typically 50 times higher than the concentrations of the chimeric strand and the complementary DNA strand.

Crystallographic space groups and approximate cell parameters for the three chimeras were determined from precession photographs (Table 1, the listed cell parameters were measured on a four-circle diffractometer). Two pictures of zero-layers of a  $[r(G)d(CGTATACGC)]_2$  crystal are shown in Figure 4. Diffraction data for the three RNA-DNA chimeras were collected on a four-circle diffractometer to resolutions between 2 and 2.25 Å. The crystal structures were found to be non-isomorphous, and the orientations of the duplexes in their respective cells were subsequently determined by the Molecular Replacement method, using an A-conformation search model. Details of the structure solution, as well as of the conformation adopted by the chimeric duplexes, are presented elsewhere<sup>22,24</sup>.

## CONCLUSIONS

In this paper we have shown that the ribonucleoside phosphoramidites **2a–d** are compatible with standard DNA phosphoramidite synthesis chemistry for the preparation of RNA-DNA chimeras of crystallographic purity following HPLC purification. The product RNA-DNA chimeras contained only the correct RNA 5'-3' linkages and were free from detectable nucleoside base or sugar modifications. The availability of these RNA-DNA chimeric species will allow detailed structural investigation of the conformational influence of the 2'-hydroxyl groups in double-stranded DNA-RNA hybrids.

## EXPERIMENTAL

### General materials and methods

The synthesis of the ribonucleoside phosphoramidites and the protocols for the chemical synthesis of RNA were performed according to the method of Scaringe *et al.*<sup>1</sup>. Ultraviolet (UV) spectra and colorimetric trityl determinations were performed on a Hewlett Packard 8451A spectrophotometer. 5 ml screw top vials with teflon lined caps were obtained from Pierce (Rockford, IL). All drying steps of the aqueous solutions in the deprotection and purification steps were performed in a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY). Electrophoretic gels were 20% acrylamide/7M urea and were run at 300–500 V using TBE buffer. The gels were visualized

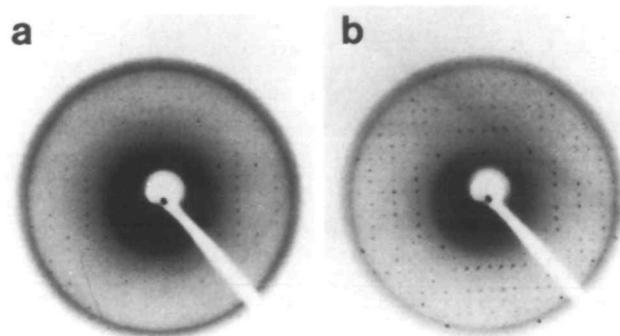


Figure 4. Precession photographs of a  $[r(G)d(CGTATACGC)]_2$  crystal: (a) (hk0) layer and (b) (h0l) layer. The precession angle was 15°, the crystal-film distance was 60 mm, and  $Cu_{K\alpha}$  radiation (Nickel filter) was used. The slight twinning of the crystal is visible from both zero-layer pictures.

and photographed by UV shadowing over a fluorescent TLC plate.

### Synthesis and deprotection of RNA-DNA chimeras

All syntheses were conducted on an ABI 380B synthesizer using the following protocol on a 10  $\mu$ mol scale. The standard 10  $\mu$ mol DNA synthesis program was used except for the ribonucleotide additions in which the wait time was extended to 10 min.

The CPG-bound chimeras were transferred from the synthesis columns to 5 ml glass screw top vials (3 per 10  $\mu$ mol synthesis). 3.5 ml of ethanolic ammonia was added to each. The vials were sealed with teflon lined caps and heated at 55°C for 16 hr. After cooling to -20°C, the ethanolic ammonia was removed from the CPG beads with silanized glass pipettes and the CPG was washed with 2  $\times$  1 ml of 50:50/EtOH:H<sub>2</sub>O which was then added to the ethanolic ammonia. The combined supernatants containing the chimeras were divided into two parts and dried to a white powder in a Speed-Vac concentrator. To remove the silyl protecting groups, each half of a synthesis was resuspended in 50  $\mu$ l of 50:50/ethanol:water and 1.2 ml of 1M TBAF/THF and left at room temperature for 24 hr. Each solution was then added directly to 10 ml of 50 mM TEAB pH 7 and loaded onto a Qiagen 500 anion exchange cartridge, prewashed with 10 ml

of 50 mM TEAB pH 7. After washing the cartridge with  $2 \times 10$  ml 50 mM TEAB pH 7, the chimera was eluted with 10 ml of 2M TEAB pH 7 and dried to a white powder.

#### Purification of fully deprotected RNA-DNA chimeras

The chimeras were first checked by analytical PAGE (0.75 mm  $\times$  20 cm  $\times$  20 cm, 1 ODU of chimera). The desired sequence was established according to electrophoretic mobility and purified by preparative HPLC on a RAININ Dynamax-300A C4 column (12  $\mu$ m particle size). The starting buffer was 100 mM triethyl ammonium acetate (TEAA, pH 6.5), and the chimeras were eluted with a gradient of 0–50% acetonitrile over 50 min. After lyophilization, the purified chimeras were dissolved in 1 ml H<sub>2</sub>O and 1  $\mu$ l removed. The 1  $\mu$ l was diluted to 800  $\mu$ l with H<sub>2</sub>O and quantitated by measurement of A<sub>260</sub>. Typical A<sub>260</sub> = 0.6–0.7 which corresponds to 480–560 ODU = 15.8–18.4 mg = 5.2–6.1  $\mu$ mol = 52–61% yield. The samples were dried again and then reconstituted as 10 mM solutions in H<sub>2</sub>O for crystal set-ups. 0.3 ODU of each were run on a 20% PAGE to verify purity (Figure 2b).

#### Crystallography

**Crystallization.** Crystals of all three RNA-DNA chimeras were grown at 18°C in sitting drops using the vapor diffusion method. The crystallization of the left-handed chimera has been described elsewhere<sup>16</sup>. In the case of the two self-complementary strands 4 and 5, the crystallization mother liquor initially contained 1.5 mM oligonucleotide (single strand concentration), and 30 mM sodium cacodylate pH 6 buffer. The concentrations of magnesium chloride and spermine tetrachloride were both  $\sim$  7.5 mM. The sitting drops were equilibrated against a reservoir of 20 ml 40% (vol/vol) 2-methyl-2,4-pentanediol. Crystals of [d(GCGT)r(A)d(TACGC)]<sub>2</sub> began to appear after about one month as rods, which were cut to a convenient size for data collection (Figure 3a). Crystals of [r(G)d(CGTATACGC)]<sub>2</sub> began to appear after about one week as elongated bipyramids with typical dimensions of 0.6  $\times$  0.3  $\times$  0.3 mm (Figure 3b). For the crystallization of the Okazaki fragment, the two strands were annealed prior to crystallization by incubating a 1:1 molar mixture of DNA and chimeric RNA-DNA solutions at 90°C, and then slowly cooling the mixture to room temperature. The crystallization mother liquor initially contained 1.5 mM Okazaki fragment, 30 mM sodium cacodylate pH 7 buffer, 7.8 mM magnesium chloride, and 75 mM spermine tetrachloride. The sitting drops were equilibrated against a reservoir of 20 ml 40% 2-methyl-2,4-pentanediol. Crystals began to appear after one month as distorted bipyramids, and grew to a maximum size of approximately 0.3  $\times$  0.3  $\times$  0.5 mm (Figure 3c). The quality of the Okazaki fragment crystals depended strongly on the pH and the spermine tetrachloride concentration. pH values lower than 7 and spermine concentrations 2 to 5 times higher than those of the oligonucleotide strands usually resulted in the formation of oil droplets or crystals which diffracted only poorly.

**Crystal Data.** Crystals of the three RNA-DNA chimeras were sealed in glass capillaries with a droplet of mother liquor, and then mounted on a HUBER 205 precession camera, attached to a RIGAKU AFC5R rotating anode X-ray generator. Space group and initial cell contents were determined from zero-layer photographs. High resolution data were then collected at various temperatures on a four-circle diffractometer equipped with a graphite monochromator.

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#### ABBREVIATIONS

Bz = benzoyl, CPG = controlled pore glass, C4 = carbon-4 chain length derivatization, DMT = dimethoxytrityl, dN = deoxyribonucleotide, HPLC = high performance liquid chromatography, *i*Bu = *i*-butyryl, LCAA-CPG = long chain alkylamine-CPG, NH<sub>4</sub>OH = 30% aqueous ammonium hydroxide, ODU = optical density unit, PAGE = polyacrylamide gel electrophoresis, rN = ribonucleotide, Si = silyl = *t*-butyldimethylsilyl, TBAF = tetra-*n*-butylammonium fluoride, TBDMS = *t*-butyldimethylsilyl, TEA = triethylamine, TEAA = triethylammonium acetate, TEAB = triethylammonium bicarbonate, TLC = thin layer chromatography, THF = tetrahydrofuran, UV = ultraviolet.

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