Synthesis and Characterization of Chimeric 2-5A-DNA Oligonucleotides

This unit describes the synthesis of chimeric oligonucleotides in which the small (2′-5′)-oligoriboadenylate activator of RNase L is covalently joined through a butanediol phosphate linker to a deoxyribooligonucleotide (Fig. 4.4.1). The products are termed "2-5A-antisense" when the deoxyribonucleotide portion of the chimera is targeted against a specific RNA. The overall synthetic strategy (see Basic Protocol 1) is based on the phosphite-triester approach to DNA/RNA synthesis (*UNITS 3.3 & 3.5*). Appropriately protected 2-cyanoethylphosphoramidite derivatives of adenosine, the butanediol linker, and the usual four deoxyribonucleosides are used for chain elongation, and the solid-phase methodology is employed with protected deoxyribonucleosides covalently linked to controlled-pore glass (CPG) through a long-chain alkyl amine (lcaa) and a succinyl moiety. 5′-Phosphorylation of the chimera completes the basic synthesis. After deprotection, the chimera is purified (see Basic Protocol 2) and characterized by a number of independent methods (see Basic Protocols 2 to 4). Two Support Protocols describe the synthesis of the central building block for the 2-5A domain of 2-5A-antisense oligonucleotide (see Support Protocol 1) and preparation of the synthon for the linker between 2-5A and the antisense oligonucleotide (see Support Protocol 2).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer.

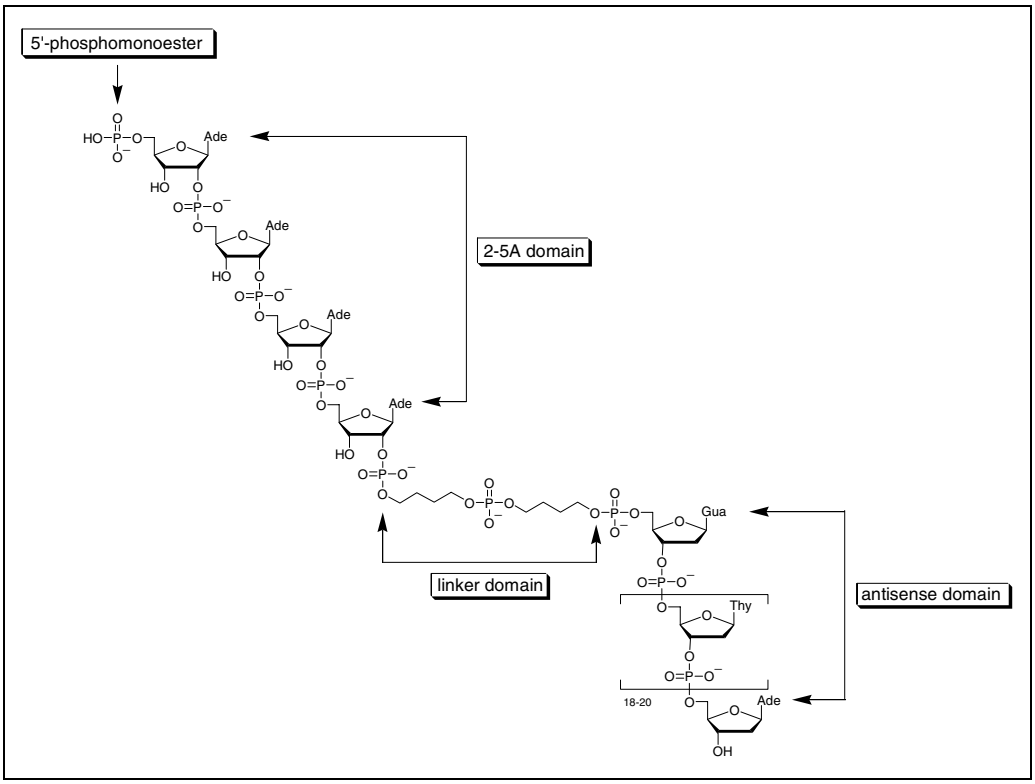

Figure 4.4.1 General structure of 2-5A-antisense oligonucleotides.

Contributed by Mark R. Player and Paul F. Torrence
Current Protocols in Nucleic Acid Chemistry (2000) 4.4.1-4.4.23

BASIC PROTOCOL 1

AUTOMATED SYNTHESIS OF CHIMERIC 2-5A-ANTISENSE OLIGONUCLEOTIDES ACCORDING TO SOLID-PHASE PHOSPHORAMIDITE CHEMISTRY

The standard 2-5A-antisense chimera has four regions (Fig. 4.4.1): (1) antisense DNA, (2) linker dimer, (3) 2-5A tetramer, and (4) 5′-terminal phosphate monoester. This procedure describes construction of the antisense domain, coupling of the butanediol linkers, addition of the (2′-5′)-oligoriboadenylate domain, and 5′-phosphorylation. Details are given for two different automated ABI DNA synthesizers. 5′-*O*-Solid supports from ABI are used and are listed below; 3′-*O*-Solid supports from Glen Research are also listed, and are used to synthesize oligonucleotides with terminal (3′-3′)-phosphodiester bonds.

Materials

Reagents for oligonucleotide synthesis:
- Acetonitrile (CH_3CN; diluent; Cruachem)
- Tetrazole/acetonitrile (activator/coupling solution; PE Biosystems)
- Acetic anhydride/lutidine/tetrahydrofuran (capping solution; PE Biosystems)
- *N*-Methylimidazole/tetrahydrofuran (capping solution; PE Biosystems)
- Trichloroacetic acid/CH_2Cl_2 (detritylation solution; PE Biosystems)
- Iodine/H_2O/pyridine/tetrahydrofuran (oxidizer; PE Biosystems)
- Tetraethylthiuram disulfide (TETD)/CH_3CN (PE Biosystems; for sulfurization)

0.1 M phosphoramidite solutions (see recipe):
- Linker CE phosphoramidite: 4-*O*-(4,4′-dimethoxytrityl)oxybutyl-1-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite] (see Support Protocol 2)
- RNA CE phosphoramidite (2-5A): N^6-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)adenosine-2′-(*N*,*N*-diisopropyl-2-cyanoethyl)phosphoramidite (see Support Protocol 1)
- Phosphorylation reagent: 2-[2-(4,4′-Dimethoxytrityl)ethylsulfonyl]ethyl-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite
- 5′-*O*-Dimethoxytrityl-N^6-benzoyl-2′-deoxyadenosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (dA^{Bz})
- 5′-*O*-Dimethoxytrityl-N^2-isobutyryl-2′-deoxyguanosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite ($dG^{i\text{-}Bu}$)
- 5′-*O*-Dimethoxytrityl-N^4-benzoyl-2′-deoxycytidine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (dC^{Bz})
- 5′-*O*-Dimethoxytrityl-2′-deoxythymidine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (T)

3:1 (v/v) concentrated NH_4OH/ethanol

1 M *n*-tetrabutylammonium fluoride (TBAF)/tetrahydrofuran (THF; Aldrich)

10 mM *n*-tetrabutylammonium phosphate (TBAP), pH 7.5, in H_2O

Automated DNA synthesizer (ABI Biotechnologies model 391 or 392) with 5′ or 3′ solid supports:
- 5′-*O*-Dimethoxytrityl-N^6-benzoyl-2′-deoxyadenosine-3′-lcaa-CPG (1 μmol; dA^{Bz}-lcaa-CPG; ABI)
- 5′-*O*-Dimethoxytrityl-N^4-benzoyl-2′-deoxycytidine-3′-lcaa-CPG (1 μmol; dC^{Bz}-lcaa-CPG; ABI)
- 5′-*O*-Dimethoxytrityl-N^2-isobutyryl-2′-deoxyguanosine-3′-lcaa-CPG (1 μmol; $dG^{i\text{-}Bu}$-lcaa-CPG; ABI)
- 5′-*O*-Dimethoxytritylthymidine-3′-lcaa-CPG (1 μmol; T-lcaa-CPG; ABI)
- 3′-*O*-Dimethoxytrityl-N^6-benzoyl-2′-deoxyadenosine-5′-lcaa-CPG (1 μmol; Glen Research)

3′-*O*-Dimethoxytrityl-N^4-benzoyl-2′-deoxycytidine-5′-lcaa-CPG (1 µmol; Glen Research)
3′-*O*-Dimethoxytritylthymidine-5′-lcaa-CPG (1 µmol; Glen Research)
3′-*O*-Dimethoxytrityl-N^2-isobutyryl-2′-deoxyguanosine-5′-lcaa-CPG (1 µmol; Glen Research).
Water bath at 55°C
Speedvac evaporator

NOTE: The above-mentioned 5′ solid supports are used in the steps below. The 3′ solid supports are used in place of the 5′ supports when synthesizing oligonucleotides with terminal (3′-3′)-phosphodiester bonds.

Synthesize 2-5A-antisense chimera

For ABI 391 DNA synthesizer:

1a. Perform automated synthesis of 2-5A-antisense oligonucleotide on an ABI 391 DNA synthesizer according to manufacturer's instructions. Use manufacturer's 1 µmol scale synthetic cycles for the antisense region and modify step 13 of the manufacturer's protocol for the remaining regions (see Table 4.4.1).

2a. Synthesize remaining regions of the chimera by sequentially adding 0.1 M solutions of linker CE phosphoramidite, RNA CE phosphoramidite (2-5A), and phosphorylation reagent. Use monomer CE phosphoramidites that are <1 week old. To change reagents, sequentially place on bottle position no. 5 as follows:

a. Remove the previous reagent and replace with 5 mL CH_3CN.
b. Remove the column feed line and dry the column by turning valve no. 14 ON for 3 min and then OFF, then turning function no. 105 ON (4 mL CH_3CN to column with the feed line unhooked) and then OFF. Replace column feed line.
c. Change bottle no. 5 to the next region's CE phosphoramidite.
d. Repeat for synthesis of each chimera domain.

For ABI 392 DNA synthesizer:

1b. Perform automated synthesis of 2-5A-antisense oligonucleotide on an ABI 392 DNA synthesizer according to manufacturer's instructions.

2b. Synthesize remaining regions of the chimera by adding linker CE phosphoramidite, RNA CE phosphoramidite (2-5A), and phosphorylation reagent using monomer

Table 4.4.1 Synthesis Conditions for Different Domains of the 2-5A-Antisense Chimera

Chimera region	Sequence no. and monomer sequence	Cycle no. and coupling time	Monomer CE phosphoramidite	Trityl mode
DNA antisense	Sequence 2 5′-(N_x)-3′	Cycle 2 15 sec	0.1 M DNA CE phosphoramidite	ON
Linker	Sequence 4 5′-(LL)(N_x)-3′	Cycle 4 300 sec	0.1 M linker CE phosphoramidite	ON
2-5A	Sequence 3 5′-(AAAA)(LLN_x)-3′	Cycle 3 600 sec	0.1 M 2′,5′-$A^{Bz\ or\ PAC}$ RNA CE phosphoramidite	ON
Phosphate	Sequence 1 5′-(p)(AAAALLN_x)	Cycle 1 60 sec	0.2 M phosphorylation reagent	OFF

positions 5, 6, and 7, respectively. Use monomer CE phosphoramidites that are <1 week old.

On the ABI 392, multiple cycle chimera sequences are created and saved on the computer's hard disk. The linker CE phosphoramidite, 2-5A CE phosphoramidite, and phosphorylation reagent are kept on monomer positions 5, 6, and 7, and need not be removed until they expire. This minimizes exposure to air. Average stepwise yield (ASWY) and overall yield are available for each synthesis.

Several cycles were created with the appropriate wait times by modification of the standard 1 µmol CE cycle (1mmol linker, 1 mmol phosphorylation). These changes are summarized in Table 4.4.2.

Cleave 2-5A-antisense oligonucleotide from CPG and deprotect it

3. Add 3 mL of 3:1 (v/v) concentrated NH_4OH/ethanol to the CPG support and incubate 2 hr at room temperature.
4. Transfer to 55°C and incubate 8 to12 hr.

   Alternatively, in the case of all phenoxyacetyl (PAC)-protected bases, incubate at room temperature for 1 hr, followed by 55°C for 1 hr.

5. Transfer solution to a test tube, cool to 0°C, and evaporate solvent in a Speedvac evaporator.
6. Remove 3′-*O*-TBDMS group from the 2-5A region of the chimera by adding 1 mL of 1 M TBAF/THF and incubating 12 to 18 hr at room temperature.

   The chimera will normally remain in solution at the end of the incubation, and the solution will be homogenous. If it is not, briefly sonicate to achieve solution and incubate for another 4 hr.

7. Add 2 mL of 10 mM TBAP, pH 7.5, and remove THF in the Speedvac evaporator.
8. Dissolve crude chimera in distilled, deionized water to a total volume of 6 mL, and purify by HPLC (see Basic Protocol 2).

   If purification cannot be performed immediately, the chimera can be stored at –70°C in the dry state for up to 2 weeks.

Table 4.4.2 Automated Synthesizer Coupling Times and Conditions

Chimera region	Monomer sequence	Cycle name and coupling time	Monomer CE phosphoramidite	Trityl mode
DNA antisense	5′-(N_x)-3′	1 µmol CE; 15 sec	0.1 M DNA CE phosphoramidite	-
Linker	5′-(LL)(N_x) -3′	1 µmol linker; 300 sec	0.1 M linker CE phosphoramidite	-
2-5A	5′-(AAAA)(LLN_x)-3′	1 µmol RNA; 600 sec	0.1 M 2′,5′-A^{Bz} RNA CE phosphoramidite	-
Phosphate	5′-(P)(AAAALLN_x)-3′	1 µmol phosphorylation; 60 sec	0.2 M phosphorylation reagent	OFF
Sulfurization	Any linkage	1 µmol phosphothioate; 25 sec, 5 sec delivery	TETD/CH_3CN bottle no. 10	-

PREPARATION OF KEY INTERMEDIATE FOR SYNTHESIS OF THE (2′,5′)-OLIGOADENYLATE (2-5A) DOMAIN

This protocol describes the procedures used to generate the appropriately protected phosphoramidite, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-N^6-benzoyl-adenosine-2′-(*N*,*N*-diisopropyl-2-cyanoethyl)phosphoramidite for adding adenosine through the formation of a 2′-5′ linkage to preformed antisense and linker domains. This phosphoramidite derivative may also be used to prepare unligated (2′-5′)-oligoadenylates using solid-phase synthesis.

Materials

Pyridine (Aldrich) dried over molecular sieves
Adenosine (Aldrich) dried overnight in vacuo over P_2O_5 (Aldrich)
4-Dimethylaminopyridine (DMAP; Aldrich)
Triethylamine (Aldrich)
4,4′-Dimethoxytrityl chloride (DMTr·Cl; Aldrich)
Chloroform ($CHCl_3$; Aldrich)
Methanol (Aldrich)
Trimethylsilyl chloride (Aldrich)
Benzoyl chloride (Aldrich)
Concentrated ammonium hydroxide (Aldrich)
Diethyl ether (Aldrich)
$MgSO_4$ (anhydrous; Aldrich)
Kieselgel 60 silica gel (220 to 440 mesh; Fluka)
Ethyl acetate (Aldrich)
Toluene (Aldrich)
Imidazole (Aldrich)
tert-Butyldimethylsilyl chloride (TBDMS·Cl; Aldrich)
N,*N*-Dimethylformamide (DMF; anhydrous; Aldrich)
Cyclohexane (Aldrich)
5% (w/v) aqueous Na_2CO_3
Methylene chloride (CH_2Cl_2; Aldrich)
1*H*-Tetrazole (Aldrich)
P_2O_5 (Aldrich)
Dry nitrogen
2-Cyanoethyl-(*N*,*N*,*N*′*N*′-tetraisopropyl)phosphoramidite (Aldrich)
Benzene

250-mL flask with ground-glass stopper
Rotary evaporator with vacuum pump
2 × 15-cm, 5 × 15-cm, and 5 × 20-cm chromatography columns
25-mL two-arm reaction flask fitted with a rubber septum
5-mL hypodermic syringe

Additional reagents and equipment for thin-layer chromatography (TLC) and fast silica gel column (flash) chromatography

Synthesize N^6-benzoyl-5′-O-DMTr-adenosine

1. Mix the following in a 250-mL flask that can be sealed to the atmosphere:

   100 mL dry pyridine

   2.67 g (10 mmol) dry adenosine

61 mg (0.5 mmol) DMAP

1.9 mL (14 mmol) triethylamine

4.1 g (10 mmol) DMTr·Cl.

Introduce a magnetic stirring bar, seal the flask with a ground-glass stopper, and stir 2 to 3 hr at room temperature.

2. Monitor the completeness of the reaction by analyzing an aliquot by silica gel TLC using 95:5 (v/v) $CHCl_3/CH_3OH$ as the eluent. For comparison, run DMAP, adenosine, and DMTr·Cl as TLC standards. Use care to completely evaporate the pyridine from the TLC plate before development.

3. When the tritylation reaction is complete, place in an ice/water bath to maintain at 0°C, and slowly add 7.7 mL (60 mmol) trimethylsilyl chloride. Incubate 15 min.

4. Allow mixture to warm to room temperature, add 5.8 mL (50 mmol) benzoyl chloride, and stir ~2 hr at room temperature.

5. Cool mixture in the ice/water bath, add 20 mL cold water, and allow to stand for 5 min.

6. Add 20 mL concentrated ammonium hydroxide (final 2 M NH_4OH), allow to warm to room temperature, and incubate 30 min at room temperature.

7. Concentrate to ~20 mL in a rotary evaporator in vacuo using a vacuum pump.

8. Add 700 mL diethyl ether, wash three times with 100 mL water, and dry the ether layer over 50 g anhydrous $MgSO_4$.

9. Filter off $MgSO_4$ and evaporate filtrate in a rotary evaporator.

10. Purify product by fast silica gel column (flash) chromatography using a 5 × 20-cm Kieselgel 60 (220 to 440 mesh) column and 2% methanol/0.2% pyridine/ethyl acetate as an eluent. Collect 5-mL fractions.

11. Analyze fractions by TLC as above, combine fractions that contain product ($R_f = 0.6$), and remove solvent in a rotary evaporator.

12. Remove last traces of residual pyridine by several additions and evaporations of toluene in 50-mL portions. Store, if necessary, dry at 4°C with a desiccant (stable at least 3 months).

This is a critical step to obtain a solid product.

The product should be obtained as a pale yellow solid foam (yield 50% to 70%): TLC R_f = 0.55 on silica gel (95:5 ethyl acetate/methanol); ^1H NMR: ($CDCl_3$) δ [ppm]: 3.34 (dd, 1H, H-5′ or 5″), 3.49 (dd, ^1H, H-5′ or 5″), 4.39 (d, 1H, H-4′), 4.50 (dd, 1H, H-3′), 4.90 (t, 1H, H-2′), 6.13 (d, 1H, H-1′), 6.74-7.60 (m, aromatic protons), 8.02 and 8.04 (s, 1H, H-2 or H-8).

Synthesize N^6-benzoyl-5′-O-DMTr-2′-O-TBDMS-adenosine and N^6-benzoyl-5′-O-DMTr-3′-O-TBDMS-adenosine

13. Dissolve the following and maintain at room temperature for 2.5 hr:

1.75 g (2.6 mmol) N^6-benzoyl-5′-*O*-DMTr-adenosine

0.544 g (8 mmol) imidazole

0.60 g (5 mmol) TBDMS·Cl

10 mL anhydrous DMF.

14. Verify that all N^6-benzoyl-5′-*O*-DMTr-adenosine has reacted by running an aliquot on a silica gel TLC plate using 95:5 (v/v) ethyl acetate/methanol or 1:1 (v/v) ethyl acetate/cyclohexane as the eluent.

15. Add 1.5 mL of 5% aqueous Na_2CO_3 at 0°C to stop the reaction. Stir 15 min at <10°C.

16. Evaporate DMF in a rotary evaporator in vacuo, dissolve the resulting residue in 50 mL CH_2Cl_2, and wash three times with 50 mL water.

17. Dry CH_2Cl_2 layer over anhydrous $MgSO_4$ and then evaporate solvent using a rotary evaporator.

18. Dissolve residue in 10 mL of 1:1 (v/v) ethyl acetate/cyclohexane containing 0.2% pyridine and apply to a 5 × 15-cm Kieselgel 60 silica gel column. Elute with 49:49:2 (v/v/v) ethyl acetate/cyclohexane/pyridine. Collect 5-mL fractions.

19. Monitor elution of reaction products using silica gel TLC with 1:1:0.004 (v/v/v) ethyl acetate/cyclohexane/pyridine.

    The first product to elute is the 2′-O-silylated isomer: N^6-benzoyl-5′-O-DMTr-2′-O-TBDMS-adenosine (R_f = 0.47). After complete elution of the 2′-O-silylated isomer, the concentration of ethyl acetate must be increased to elute the 3′-O-silylated isomer.

20. Switch eluent to ethyl acetate/cyclohexane/pyridine 66:32:2 (v/v/v) and continue TLC monitoring for elution of the 3′-*O*-silylated isomer.

    N^6-Benzoyl-5′-O-DMTr-3′-O-TBDMS-adenosine has an R_f of 0.24.

21. Pool appropriate fractions containing each of the respective products and remove the solvent by rotary evaporation in vacuo. Subsequently add and evaporate 50-mL portions of toluene to remove traces of pyridine as judged by smell. Store, if necessary, dry at 4°C with a desiccant (stable at least 3 months).

    The products are obtained as colorless foams (typical yields: 480 mg 2′-O-silylated isomer; 660 mg 3′-O-silylated isomer). By silica gel TLC using 1:1:0.004 ethyl acetate/cyclohexane/pyridine, the 2′-isomer has a R_f = 0.47 and the 3′-isomer has a R_f = 0.24. The product used for further synthesis of 2-5A and 2-5A-antisense oligonucleotide is the 3′-O-silylated isomer: 1H NMR: ($CDCl_3$), δ (ppm): 0.02 and 0.10 (ds, 6H, CH_3Si), 0.98 (s, 9H, t-butyl), 3.27 and 3.54 (dd, 1H, H-5′ or H-5″), 3.77 (s,3H, CH_3O), 4.21 (m, 1H, H-4′), 4.60 (t, 1H, H-3′), 4.80 (t,1H, H-2′), 6.10 (d, 1H, H-1′), 8.03 and 8.05 (s, 1H, H-2 or H-8), 6.78-7.60, 8.28, 8.77 (m, aromatic protons).

    It is critical that complete separation of the silylated isomers be accomplished at this stage. Separation will be impossible after generation of the phosphoramidite. This is the most common cause of difficulty in this procedure.

Synthesize N^6-benzoyl-5′-O-DMTr-3′-O-TBDMS-adenosine-2′-(N,N-diisopropyl-2-cyanoethyl)phosphoramidite

22. Combine 48 mg (0.68 mmol) of 1*H*-tetrazole and 540 mg (0.68 mmol) of N^6-benzoyl-5′-*O*-DMTr-3′-*O*-TBDMS-adenosine in a 25-mL two-arm reaction flask fitted with a rubber septum and a hypodermic needle.

    For this reaction, additional care must be taken to ensure anhydrous reaction conditions. The hypodermic needle is used for evacuation of air and admission of dry nitrogen and reagents.

23. Place the flask, fittings, and contents in a vacuum desiccator and dry overnight in vacuo in the presence of P_2O_5.

24. Admit dry nitrogen to the desiccator and thus to the reaction flask. Immediately remove the hypodermic needle from the rubber septum.

25. Add a solution of 5 mL CH_2Cl_2 and 206 mg (0.68 mmol) of 2-cyanoethyl-(*N*,*N*,*N*′,*N*′-tetraisopropyl)phosphoramidite through the rubber septum using a 5-mL hypodermic syringe and needle.

26. Incubate 4 hr at room temperature and then overnight at 4°C.

27. Evaporate reaction mixture in vacuo on a rotary evaporator.

28. Add 2 mL of 6:3:1 (v/v/v) benzene/cyclohexane/triethylamine to the residue and apply to a 2 × 15-cm silica gel (Kieselgel 60) column. Use the same solvent for product elution. Collect 5-mL fractions.

29. Monitor fractions by silica gel TLC using 6:3:1 (v/v/v) benzene/cyclohexane/triethylamine as eluent.

    Two major products are generated in this reaction, namely, the two P-chiral diastereomers. These two stereoisomers have R_f values of 0.61 and 0.53 by silica gel TLC with this solvent.

30. Combine product-containing fractions, concentrate to dryness in vacuo, and dry by addition and evaporation of 50-mL portions of toluene.

    The product can be stored at 4°C.

    A partial separation of these two diastereomers can be achieved, but in practice this is not necessary. The product, consisting of a mixture of both P-chiral diastereoisomers, is obtained as a colorless foam (~500 mg); ^{31}P NMR: ($CDCl_3$, 1% C_5D_5N):δ (ppm) 150.73 and 150.38.

SUPPORT PROTOCOL 2

PREPARATION OF A LINKER INTERMEDIATE THAT JOINS (2′-5′)-OLIGOADENYLATE (2-5A) TO ANTISENSE (3′-5′)-OLIGODEOXYRIBONUCLEOTIDES

This protocol describes the synthesis of 4-*O*-(4,4′-dimethoxytrityl)oxybutyl-1-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite], which is the moiety used in the prototype 2-5A-antisense chimera to join the 2-5A domain to the antisense domain.

Materials

1,4-Butanediol (Aldrich)
Pyridine (anhydrous; Aldrich)
Dry argon or nitrogen (Aldrich)
4,4′-Dimethoxytrityl chloride (DMTr·Cl; Aldrich)
Chloroform (Aldrich)
Methanol (Aldrich)
Ethyl acetate (Aldrich)
$MgSO_4$ (anhydrous; Aldrich)
Kieselgel 60 silica gel (Fluka)
Methylene chloride (CH_2Cl_2; Aldrich)
Ethyldiisopropylamine (Aldrich)
2-Cyanoethyl-*N*,*N*-diisopropylphosphoramidic chloride (Aldrich)
Benzene (Aldrich)
Petroleum ether (Aldrich)
Triethylamine (Aldrich)

250-ml stoppered flask
Rotary evaporator attached to a vacuum pump
Hand-held UV lamp

Additional reagents and equipment for thin-layer chromatography (TLC) and fast silica gel column (flash) chromatography

Synthesize 4-O-(4,4′-dimethoxytrityl)oxybutan-1-ol

1. Dissolve 9.0 g (100 mmol) of 1,4-butanediol in 50 mL anhydrous pyridine in a 250-ml stoppered flask.
2. Remove pyridine by evaporation at <40°C in a rotary evaporator attached to a vacuum pump.
3. Through the rotary evaporator stopcock, admit dry argon or nitrogen to the flask containing the butanediol, add an additional 50 mL dry pyridine, and repeat the evaporation procedure.
4. Repeat step 3 once more. Be certain to admit the dry nitrogen or argon after the final pyridine evaporation.

   A total of three evaporations will dry the butanediol sufficiently for the following tritylation reaction.

5. Dissolve dried 1,4-butanediol in 50 mL anhydrous pyridine.
6. Add 3.39 g (10 mmol) DMTr·Cl and allow the homogenous mixture to react 2 hr at room temperature in the stoppered flask.
7. Verify complete formation of the product by TLC using 99:1 (v/v) chloroform/methanol as developing solvent.
8. Pour the entire mixture onto 100 g ice in a beaker. Add a magnetic stirring bar and stir the mixure until the ice is completely melted.
9. Add 100 mL ethyl acetate and shake to extract the organic product. Separate the organic (top) and aqueous (bottom) layers.
10. Reextract the aqueous layer with an additional 50 mL ethyl acetate. Separate the organic layer again, and add the ethyl acetate layer to the one obtained from the previous extraction.
11. Add 10 g anhydrous $MgSO_4$ to the combined organic layers to dry them. Filter off $MgSO_4$ and evaporate all but ~10 mL ethyl acetate solution in a rotary evaporator at <40°C.
12. Add the concentrated ethyl acetate solution to the top of a column containing 250 g silica gel (Kieselgel 60). Elute with 99:1 (v/v) methylene chloride/methanol, collecting 10-mL fractions.
13. Check each fraction for product by spotting aliquots on a silica gel TLC plate and looking for the presence of UV-absorbing material with a hand-held UV lamp. If UV absorbance is detected, develop the plate using 99:1 (v/v) chloroform/methanol.

    Approximately 2.1 g (54% overall yield) of 4-O-(4,4′-dimethoxytrityl)oxybutan-1-ol can be obtained: ^1H NMR ($CDCl_3$, 1% deuteriopyridine):δ (ppm) 1.68(m, 4H, CH_2); 3.10 (t, J = 5.7 Hz, 2H, CH_2O); 3.62 (t, J = 5.8 Hz, 2H, CH_2OH); 3.76 (s, 6H, CH_3O); 6.79-7.46 (m, aromatic protons).

Synthesize 4-O-(4,4′-dimethoxytrityl)oxybutyl-1-[(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite]

14. Prepare a solution of the following reagents under anhydrous conditions:

    390 mg (1 mmol) 4-*O*-(4,4′-dimethoxytrityl)oxybutan-1-ol

510 mg (4 mmol) ethyldiisopropylamine

3 mL dry methylene chloride.

Cool in an ice bath.

15. Slowly add 237 mg (1 mmol) of 2-cyanoethyl-*N,N*-diisopropylphosphoramidic chloride under anhydrous conditions. Allow to warm to room temperature and to react for 1 hr.

16. Evaporate solvent using a rotary evaporator at <40°C.

17. Dissolve residue in 5 mL benzene and add mixture to the top of a 1.8 × 14-cm silica gel column. Elute the product with 6:3:1 (v/v/v) benzene/petroleum ether/triethylamine.

18. Check fractions for product by silica gel TLC using 6:3:1 (v/v/v) benzene/petroleum ether/triethylamine as the eluent.

19. Evaporate solvent from product-containing fractions.

Yield: 580 mg (98%) 4-O-(4,4′-dimethoxytrityl)oxybutyl-1-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]: ^1H NMR ($CDCl_3$, 1% deuteriopyridine):δ (ppm) 1.17 (t, J = 7.0 Hz, 12 H, CH_3C); 1.70 (m, 4H, CH_2C); 2.60 (t, J = 6.5 Hz, 2 H, CH_2CN), 3.08 (t, J = 5.7 Hz, 2H, CH_2O); 3.80 (m, 2H, CH); 6.80-7.49 (m, aromatic protons); ^{31}P NMR ($CDCl_3$, 1% deuteriopyridine):δ (ppm) 147.6.

This phosphoramidite, like all phosphoramidites, is best stored in the presence of a desiccant at –20°C.

BASIC PROTOCOL 2

PURIFICATION AND CHROMATOGRAPHIC CHARACTERIZATION OF CHIMERIC 2-5A ANTISENSE OLIGONUCLEOTIDES

After deprotection, the chimera can be purified using either reversed-phase ion-pair chromatography or anion-exchange chromatography. The polystyrene reversed-phase ion-pair (PRP-1) column has a long lifetime, can tolerate extremes of pH and fluoride ions, and gives good recoveries of the 2-5A-antisense chimera. Desalting prior to ion-pair HPLC is not required, but is performed after. A typical chromatogram of a representative crude chimera is shown in Figure 4.4.2. The Nucleogen DEAE anion-exchange column has a shorter lifetime than the PRP-1 column, and cannot tolerate extremes of pH and fluoride ions. Preparative purification requires prior desalting. In addition, recoveries of chimera are poorer than those accomplished with the PRP-1 procedure. After purification by ion-pair or anion-exchange chromatography, the chimera is converted to a sodium salt by cation exchange, dialyzed, and filter sterilized. The purity of the chimera is estimated by capillary gel electrophoresis and ion-exchange HPLC.

Materials

Crude 2-5A-antisense chimera (see Basic Protocol 1)
Solvent A: 10 mM *n*-tetrabutylammonium phosphate (TBAP), pH 7.5, in H_2O (for ion-pair chromatography)
Solvent B: 10 mM TBAP, pH 7.5, in 8:2 (v/v) CH_3CN/H_2O (for ion-pair chromatography)
Methanol
Solvent C: 20 mM potassium phosphate, pH 7.0, in 8:2 (v/v) CH_3CN/H_2O (for anion-exchange chromatography)
Solvent D: 20 mM potassium phosphate, pH 7.0, in aqueous 1 M KCl (for anion-exchange chromatography)
Dowex 50W (Na^+ form; Bio-Rad)
Tris/methanol running buffer: 75 mM Tris phosphate, pH 7.6, in 9:1 (v/v) H_2O/methanol

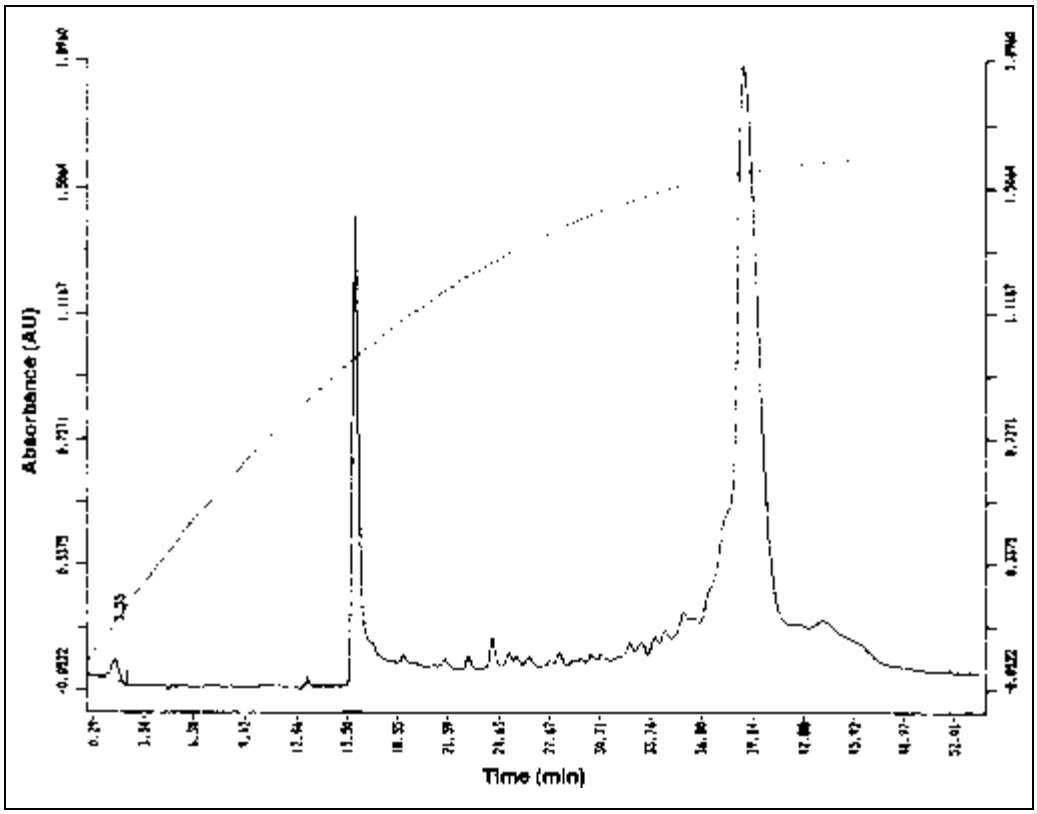

Figure 4.4.2 Polystyrene reversed-phase ion-pair chromatography of the crude (post-deprotection) 2-5A-DNA chimera: 5′-pA(2′,5′-pA)$_3$-2′-p[$(CH_2)_4$p]$_2$d[TCT CCG CTT CTT CCT GCC AT]. A 33 × 7-mm PRP-1 column was used and elution was accomplished with a convex gradient of 5% to 80% solvent B in solvent A over 50 min, where solvent A was 10 mM *n*-tetrabutylammonium phosphate (TBAP), pH 7.5, and solvent B was 10 mM TBAP in 8:2 (v/v) methanol/water, pH 7.5. The flow rate was 1.5 mL/min. Detection was at 270 mm. The major peak at a retention time of ~39 min was collected.

Solvent E: 25 mM Tris·Cl, pH 7.0 *(APPENDIX 2A)* in 1:200 (v/v) CH_3CN/H_2O
Solvent F: 25 mM Tris·Cl, 1 M ammonium chloride, pH 7.0, in 1:200 (v/v) CH_3CN/H_2O

High-performance liquid chromatograph (HPLC) with 270-nm UV detection
300 × 7-mm polystyrene reversed-phase column (PRP-1 semi-prep column; 10 µm, 100 Å; Hamilton; for ion-pair chromatography)
Speedvac evaporator
SepPak C18 cartridge
125 × 4–mm Nucleogen DEAE 60-7 column (7 µm, 60 Å; Macherey-Nagel; for anion-exchange chromatography)
PolyPrep column
Millex-GV 0.22-µm filter unit (Millipore)
SpectraPor dialysis chamber (V_t = 5 mL) with a 3500 MWCO membrane (Spectrum)
Capillary electrophoresis instrument (ABI model 270A-HT) with MICRO-GEL_{100} gel-filled capillaries (50-µm i.d., 27-cm effective length) and UV detection at 260 nm
250 × 4-mm Dionex PA-100 column (Dionex)

Additional reagents and equipment for determining OD_{260} (*UNIT 10.3*)

Purify 2-5A-antisense chimera

For reversed-phase ion-pair chromatography:

1a. Inject 1 mL crude 2-5A-antisense chimera into a 300 × 7–mm polystyrene reversed-phase column.

2a. Elute purified chimera using a convex gradient from 5:95 (v/v) solvents B/A to 80:20 solvents B/A over 45 min, at a flow rate of 1.5 mL/min. Collect 1-mL fractions and use UV detection at 270 nm.

3a. Combine fractions according to the UV profile, and evaporate to dryness in a Speedvac evaporator.

4a. Wash a SepPak C18 cartridge sequentially with 10 mL methanol and 10 mL H_2O to remove shipping buffer.

5a. Dissolve chimera in 2 to 4 mL H_2O and load onto the cartridge.

6a. Wash cartridge sequentially with 15 mL H_2O, 10 mL of 5:95 (v/v) methanol/H_2O, and 10 mL of 1:9 (v/v) methanol/H_2O.

7a. Elute chimera with 10 mL of 1:1 (v/v) methanol/H_2O and concentrate the eluate as needed in a Speedvac evaporator. Proceed to step 8.

For anion-exchange chromatography:

1b. Desalt crude 2-5A-antisense chimera as described in steps 4a to 7a.

2b. Inject 1 mL chimera in water onto a 125 × 4–mm Nucleogen DEAE 60-7 column.

3b. Elute purified chimera using a linear gradient from 1:99 (v/v) solvents D/C to 100% solvent D over 30 min, followed by isocratic elution for 15 min, all at a flow rate of 1.0 mL/min. Collect 1-mL fractions and use UV detection at 260 nm.

4b. Combine fractions as indicated by the UV profile, and evaporate to dryness in a Speedvac evaporator. Proceed to step 8.

Convert chimera to its sodium salt by cation exchange

8. Dissolve tetrabutylammonium salt (step 7a) or potassium salt (step 4b) of the chimera in 2 mL water.

9. Add 1 mL Dowex 50W slurry (Na^+ form) and stir 3 hr at 4°C.

10. Remove resin by passing the suspension through an empty PolyPrep column.

11. Elute chimera from the resin with 17 mL H_2O. Be sure to monitor the recovery by UV, as the oligonucleotide is retarded by the Dowex.

Dialyze and sterilize chimera

12. Sterilize solution through a Millex-GV 0.22-µm filter unit.

13. Dialyze against water overnight at 4°C, using a SpectraPor dialysis chamber (V_t = 5 mL) with a 3500 MWCO membrane.

14. Repeat step 12. Determine OD_{260} (*UNIT 10.3*) to quantitate DNA.

These steps are necessary for removal of low-molecular-weight impurities and to prepare the chimera for biological evaluation.

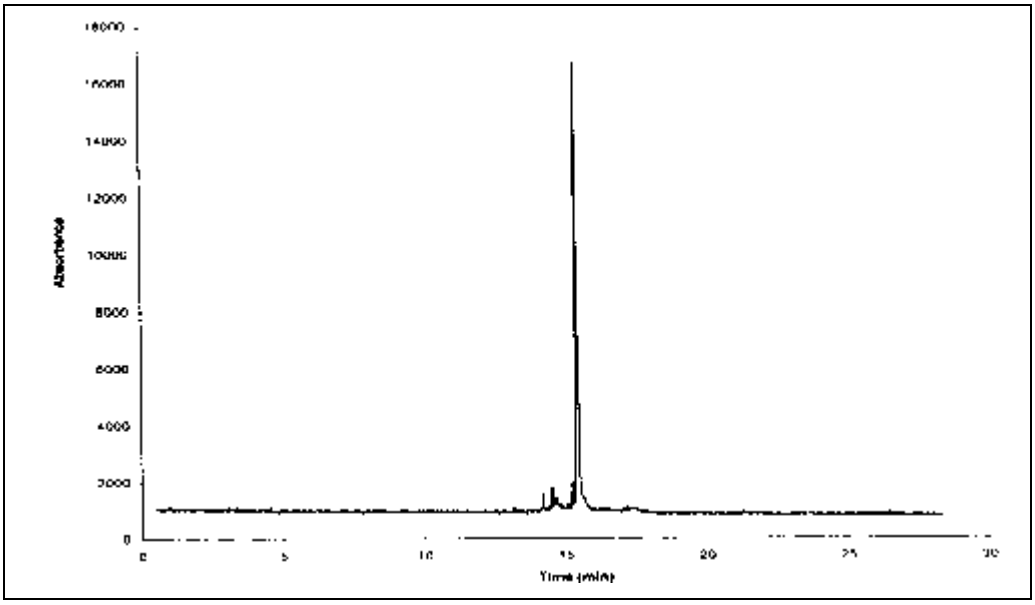

Figure 4.4.3 Determination of 2-5A-antisense chimera purity by capillary gel electrophoresis on an ABI 270A-HT instrument using MICRO-GEL$_{100}$ gel-filled capillaries (50-μm i.d., 27-cm effective length) with a running buffer of 75 mM Tris phosphate, pH 7.6, in 9:1 (v/v) H_2O/methanol. The voltage was –14 kV and the operation *T* was 30°C. Detection was at 260 nm. The typical chromatogram shown is for the chimera: 5′-pA(2′,5′-pA)$_3$-2′-p[$(CH_2)_4$p]$_2$d[TCT CCG CTT CTT CCT GCC AT].

Estimate chimera purity by capillary gel electrophoresis

15. Determine oligonucleotide chimera purity on an ABI 270A-HT capillary electrophoresis instrument using MICRO-GEL$_{100}$ gel-filled capillaries and Tris/methanol running buffer with UV detection at 260 nm. Run at –14 kV (17 μA) and at an operation temperature of 30°C.

    Typically, a chimera electropherogram can be obtained using a sample concentration of 0.06 OD_{260} and an electrokinetic injection of 2 s at –5 kV. A typical electropherogram is shown in Figure 4.4.3.

Estimate chimera purity by ion-exchange HPLC

16. Carry out ion-exchange HPLC using a 250 × 4–mm Dionex PA-100 column. Use an injection volume of 50 μL and ~0.1 OD_{260} of oligonucleotide. Employ a linear gradient from 1:9 (v/v) solvents F/E to 9:1 solvents F/E over 25 min, followed by isocratic elution for 25 min, all at a flow rate of 1.0 mL/min with UV detection at 270 nm.

    The Dionex PA-100 column gives better peak shape than many other ion-exchange columns.

COMPOSITION ANALYSIS OF CHIMERIC 2-5A-ANTISENSE OLIGONUCLEOTIDES USING SNAKE VENOM PHOSPHODIESTERASE DIGESTION AND REVERSED-PHASE HPLC

BASIC PROTOCOL 3

When 2-5A-antisense chimeric oligonucleotides are digested by snake venom phosphodiesterase (SVPD), they yield 5′-AMP from the 2-5A domain, 5′-pA2′-p$(CH_2)_4$p$(CH_2)_4$OH from the terminal AMP of 2-5A and the linker moiety, and deoxyribonucleotide 5′-monophosphates from the antisense domain. This analysis provides a cornucopia of valuable information regarding the 2-5A-antisense chimera. It provides a compositional analysis (by providing the ratio of 5′-AMP to any or all of the constituent 5′-deoxyribonucleotides) and

also ascertains the completeness of 5′-phosphorylation (through the appearance of adenosine as a product of 5′-unphosphorylated chimera), which is vital for full activation of RNase L.

Materials

Purified 2-5A-antisense chimera (see Basic Protocol 2)
Snake venom phosphodiesterase (SVPD, *Crotallus adamanteus*; Amersham Pharmacia Biotech)
1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 M $MgCl_2$
Solvent A: aqueous 100 mM ammonium phosphate, pH 5.5
Solvent B: 1:1 (v/v) methanol/H_2O

Microcon-10 concentrator (Amicon)
High-performance liquid chromatograph (HPLC) with 250 × 4.6–mm Ultrasphere C18 ODS column (5 µm, 80 Å; Thomson Instrument)

1. Analyze the nucleotide composition of purified 2-5A-antisense chimera by digestion with SVPD. Mix the following and incubate 3 hr at 37°C:

   0.2 OD_{260} units chimera

   0.15 units SVPD

   50 mM Tris·Cl, pH 8.0

   0.5 mM $MgCl_2$

   water to 100 µL.

2. Wash the membrane of a Microcon-10 concentrator with 100 µL water and then with 100 µL solvent A to remove shipping buffer. Centrifuge each wash 5 min at 700 × *g*, 4°C (see manufacturer's instructions).

   Each spin-rinse takes ~15 min.

3. Discard filtrate, apply 100 µL enzyme digestion mixture, and spin-rinse for 15 min.

4. Wash membrane three more times with 100-µL portions of solvent A to ensure that all nucleotides have passed through the membrane.

5. Analyze the centrifugate by reversed-phase HPLC using a 250 × 4.6–mm Ultrasphere C18 ODS column. Inject 20 to 30 µL. Separate the digestion products using the following solvent program at a flow rate of 0.5 mL/min:

   a. isocratic elution with 1:99 (v/v) solvents B/A for 20 min

   b. linear gradient elution from 1:99 solvents B/A to 45:55 solvents B/A over 30 min

   c. isocratic elution with 45:55 solvents B/A for 20 min.

   Typical retention times for various nucleotides are: 5′-dCMP (ε_{260} = 7,610), 9.7 min; 5′-TMP (ε_{260} = 8,158), 27.3 min; 5′-dGMP (ε_{260} = 9,969), 29.6 min; 5′-AMP (ε_{260} = 12,300), 31.7 min; 5′-dAMP (ε_{260} = 14,300), 41.2 min; 5′-pA2′-p$(CH_2)_4$p$(CH_2)_4$OH [AMP(pBu)$_2$] (ε_{260} = 12,300), 39.5 min. A typical chromatogram of a digestion is shown in Figure 4.4.4.

6. Normalize the absorbance of each peak by dividing by the extinction coefficient (ε_{260}), and thereby ascertain the relative ratios of each nucleotide.

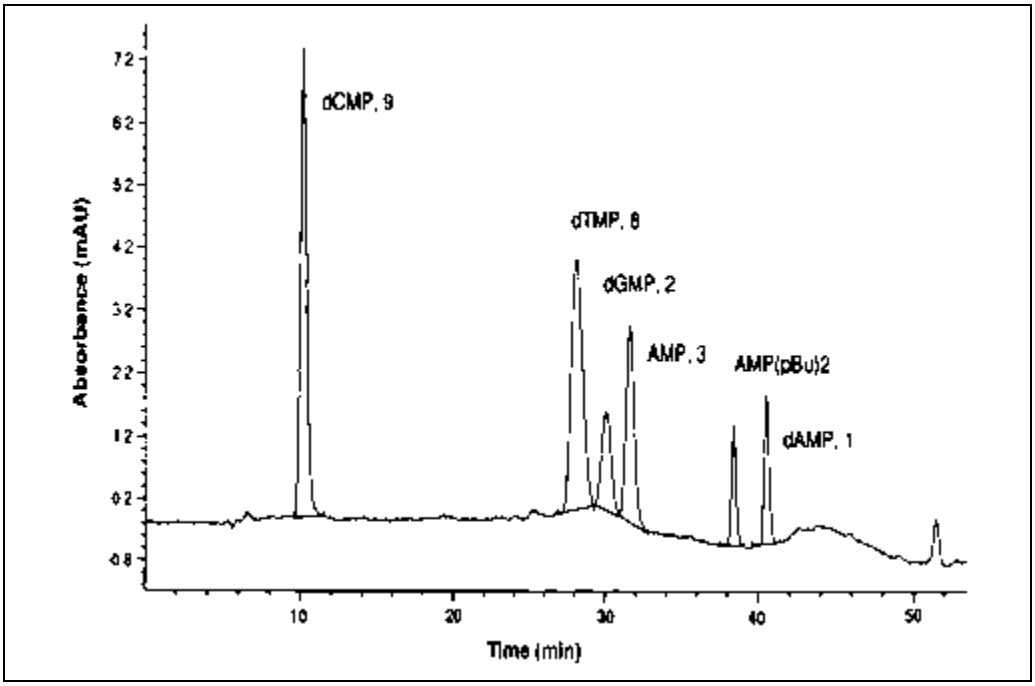

Figure 4.4.4 Digestion of a representative 2-5A-antisense chimera with snake venom phosphodiesterase and analysis by HPLC. A snake venom phosphodiesterase digest of the chimera 5′-pA(2′,5′-pA)$_3$-2′p[(CH$_2$)$_4$p]$_2$d[TCT CCG CTT CTT CCT GCC AT] was injected into a 250 × 4.6-mm Beckman Ultrasphere C18 ODS column. Elution was with 2% solvent B in 98% solvent A isocratically for 20 min, then a linear gradient of 2% to 50% solvent B in solvent A for 15 min, then 50% solvent B isocratically for 20 min. Solvent A was 100 mM ammonium phosphate, pH 5.5, and solvent B was 1:1 (v/v) methanol/H$_2$O. The flow rate was 0.5 mL/min and detection was at 260 nm.

SEQUENCING CHIMERIC 2-5A-ANTISENSE OLIGONUCLEOTIDES ACCORDING TO A MODIFIED MAXAM-GILBERT PROCEDURE

BASIC PROTOCOL 4

Although the size and unusual chemical structure of 2-5A-antisense chimeras do not permit enzymatic sequencing, the Maxam-Gilbert procedure can be employed if certain modifications are followed. In this procedure, the chimera is 3′-end labeled using [α-^{32}P]ddATP and terminal nucleotide transferase.

Materials

Purified 2-5A-antisense chimera (see Basic Protocol 2)
5× TNT buffer (see recipe)
[α-^{32}P]ddATP (3000 Ci/mmol, 10 mCi/mL; Amersham)
15 U/μL terminal deoxyribonucleotide transferase (TNT) from calf thymus (Life Technologies)
0.5 M EDTA, pH 8 (*APPENDIX 2A*)
1 mg/mL calf thymus DNA (Clontech)
0.5 M sodium phosphate buffer, pH 6.8 (see recipe)
Scintillation fluid
Diethyl pyrocarbonate (DEPC; Sigma)
DEPC buffer (see recipe)
Ethanol
0.3 and 3 M sodium acetate (Quality Biological), pH 6, in water
4 mg/mL yeast tRNA (Clontech)
Dimethyl sulfate (DMS; Aldrich)
DMS buffer (see recipe)
DMS stop buffer (see recipe)

A>C buffer (see recipe)
1 N acetic acid
Hydrazine (HZ; Aldrich)
HZ stop buffer (see recipe)
5 M NaCl (Quality Biological) in water
1 M piperidine (Aldrich) in water, freshly prepared
Denaturing gel-loading buffer (see recipe)
10× TBE electrophoresis buffer (*APPENDIX 2A*)

Lyophilizer
ChromaSpin-10 gel column (Clontech)
Whatman DE81 filters
Vacuum manifold
Siliconized tubes
Heating blocks at 25°, 60°, and 90°C

Additional reagents and equipment for polyacrylamide gel electrophoresis (PAGE; *APPENDIX 3B*)

3′-End label 2-5A-antisense chimera

1. Lyophilize 10 pmol of purified 2-5A-antisense chimera.
2. Add the following in order:

   10 µL 5× TNT buffer

   32 µL ddH_2O

   6 µL [α-^{32}P]ddATP (20 pmol)

   2 µL 15 U/µL TNT.
3. Incubate 60 min at 37°C.
4. Stop reaction by adding 4 µL of 0.5 M EDTA, pH 8, and 6 µL of 1 mg/mL calf thymus DNA. Vortex gently.
5. Set aside 1 µL for determination of specific activity (steps 8 to 11).
6. Apply the remainder of the reaction to a precentrifuged ChromaSpin-10 gel column and elute with water by centrifuging 6 min at 700 × *g*, at 4°C.
7. Lyophilize eluted solution and store at –20°C until use (up to 2 weeks).

Determine specific activity of labeled chimera

8. Dilute 1 µL labeling reaction (from step 5) in 99 µL of 0.2 M EDTA, pH 8.
9. Place quadruplicate 3-µL spots of diluted sample on separate Whatman DE81 filters. Dry at 50°C.
10. Wash two filters on a vacuum manifold with 30 mL of 0.5 M sodium phosphate buffer, pH 6.8, and dry them at 50°C.

    Washing will remove unincorporated nucleotide. The two unwashed filters are used to determine the total cpm in the sample.
11. Add scintillation fluid to each filter and count them in a liquid scintillation counter. Use the cpm from the filters to determine the following:

    % incorporation = cpm incorporated/total cpm

% 3′ ends labeled = % incorporation × 2

specific activity = % incorporation × total cpm added to reaction/μg chimera.

Expected values are 10% to 15% incorporation, with 20% to 30% of 3′ ends labeled, and specific activity of ~10^8 cpm/μg chimera.

Perform sequencing reactions

12. Set up rA reaction to cleave primarily at riboadenosine residues:
    a. Place a siliconized tube containing 1/6 of the labeled chimera on ice.
    b. Add 150 μL DEPC buffer followed by 2 μL of 1:9 (v/v) DEPC/ethanol.
    c. Vortex and incubate 5 min at 90°C.
    d. Add 15 μL of 3 M sodium acetate, pH 6.
    e. Add 5 μL of 4 mg/mL yeast tRNA and 800 μL ethanol.
    f. Vortex and bring to –70°C.
13. Set up G reaction for primary cleavage at deoxyguanosine and minor cleavage at riboadenosine.
    a. Place a siliconized tube containing 1/6 of the labeled chimera on ice.
    b. Add 200 μL DMS buffer followed by 1 μL dimethyl sulfate.
    c. Vortex and incubate 20 min at 25°C.
    d. Add 50 μL DMS stop buffer and 800 μL ethanol.
    e. Vortex and bring to –70°C.
14. Set up A>C reaction for cleavage primarily at deoxyadenosine, with secondary cleavage at deoxycytidine and riboadenosine.
    a. Place a siliconized tube containing 1/6 of the labeled chimera on ice.
    b. Add 100 μL A>C buffer.
    c. Vortex and incubate 15 min at 90°C.
    d. Add 150 μL of 1 N acetic acid, 5 μL of 4 mg/mL yeast tRNA, and 800 μL ethanol.
    e. Vortex and bring to –70°C.
15. Set up A+G reaction for cleavage at riboadenosine, deoxyadenosine, and deoxyguanosine.
    a. Place a siliconized tube containing 1/6 of the labeled chimera on ice.
    b. Add 150 μL DEPC buffer followed by 2 μL of 1:9 (v/v) DEPC/ethanol.
    c. Vortex and incubate 5 min at 90°C.
    d. Add 15 μL of 3 M sodium acetate, pH 6.
    e. Add 5 μL of 4 mg/mL yeast tRNA and 800 μL ethanol.
    f. Vortex and bring to –70°C.
16. Set up T+C reaction for cleavage at deoxythymidine, deoxycytidine, and riboadenosine.
    a. Place a siliconized tube containing 1/6 of the labeled chimera on ice.
    b. Add 15 μL water followed by 30 μL hydrazine.
    c. Vortex and incubate 60 min at 25°C.
    d. Add 400 μL HZ stop buffer and 800 μL ethanol.
    e. Vortex and bring to –70°C.
17. Set up C reaction for strong cleavage at deoxycytidine and riboadenosine.

a. Place a siliconized tube containing 1/6 of the labeled chimera on ice.
b. Add 15 µL of 5M NaCl followed by 30 µL hydrazine.
c. Vortex and incubate 60 min at 25°C.
d. Add 400 µL HZ stop buffer and 800 µL ethanol.
e. Vortex and bring to –70°C.

Wash all samples

18. Centrifuge 15 min at 12,000 × *g*, 4°C.
19. Withdraw and discard supernatant, checking with a survey meter that very little labeled chimera is removed.
20. Redissolve chimera in 400 µL of 0.3 M sodium acetate, pH 6.
21. Add 800 µL ethanol, vortex, bring to –70°C, and centrifuge 15 min at 12,000 × *g*, 4°C.
22. Repeat steps 19 and 21 (do not add sodium acetate).
23. Remove most of the supernatant and lyophilize pellet for 15 min.

Cleave modified bases with piperidine

24. Add 100 µL of 1 M piperidine to DNA samples and vortex.
25. Make sure lids are securely shut and incubate 20 min at 60°C (for rA reaction) or 30 min at 90°C (for all other reactions).
26. Cool on ice and lyophilize for 15 min.
27. Add 15 µL water, vortex, and lyophilize again. Repeat.

Perform polyacrylamide gel electrophoresis (PAGE)

28. Add 100 µL denaturing gel-loading buffer.
29. Denature samples 60 min at 90°C and then cool on ice.
30. Pour and set up 20% polyacrylamide/8 M urea sequencing gel (see *APPENDIX 3B*) using 1× TBE electrophoresis buffer and pre-run for 2 hr at 130 W constant power.

    Gel will heat to 55°C.

31. Load samples and run at 130 W constant power until the bromphenol blue has migrated ~19 cm down from the wells. Remove one or both plates.
32. Mark the upper left corner of the gel with radioactive ink and autoradiograph. A typical result is shown in Figure 4.4.5.

REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

A>C buffer

0.480 mg sodium hydroxide (1.2 N)
20 µL 1 mM EDTA
H_2O to 10 mL

Aniline solution, 1 M, pH 4.5

931 µL aniline (Aldrich)
8.869 mL H_2O

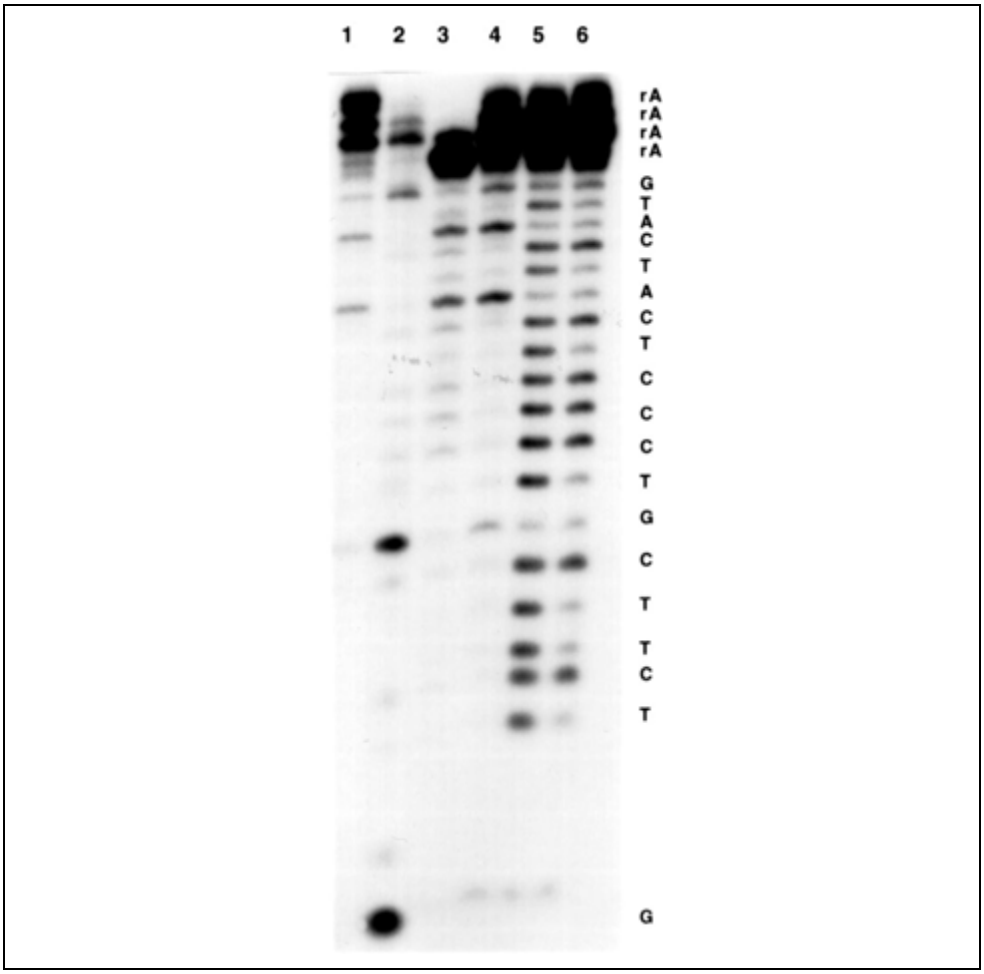

Figure 4.4.5. Maxam-Gilbert sequencing of the 2-5A-antisense chimera 5′-pA(2′,5′-pA)$_3$-2′-p[(CH$_2$)$_4$p]$_2$d[TCT CCG CTT CTT CCT GCC AT]. Lane 1, rA; lane 2, G; lane 3, A>C; lane 4, A+G; lane 5, T+C; lane 6, C.

Adjust pH to 4.5 with acetic acid
Store in the dark at –20°C

Precipitate will clear upon addition of acetic acid.

ATP/dTTP stock solution

4.8 mg dTTP (final 1.0 mM)
0.55 mg ATP (final 0.1 mM)
Adjust pH to 7.0
Add H_2O to 10.0 mL

Denaturing gel-loading buffer

8 mL formamide (Fluka; 80% v/v)
1 g sucrose (10% w/v)
40 µL 2 mM EDTA
0.2% (w/v) bromphenol blue (Sigma)
Add H_2O to 10 mL

Diethyl pyrocarbonate (DEPC) buffer

166 µL 3 M sodium acetate, pH 6 (final 50 mM, pH 5)
20 µL 0.5 M EDTA (final 1 mM)
H_2O to 10 mL

Dimethyl sulfate (DMS) buffer

80 mg sodium cacodylate (Aldrich), pH 8.0 (final 50 mM)
20 µL 1 mM EDTA
H_2O to 10 mL

DMS stop buffer

829 µL 1.5 M sodium acetate, pH 7.0
71 µL 1 M 2-mercaptoethanol
25 µL 100 µg/mL yeast tRNA
75 µL H_2O
Prepare fresh before use

Hydrazine (HZ) stop buffer

400 µL 1.5 M sodium acetate, pH 7 (final 0.3 M)
200 µL 1 mM EDTA (final 0.1 mM)
15 µL 25 µg/mL yeast tRNA (final 0.2 µg/mL)
H_2O to 2 mL
Prepare fresh before use

Phosphoramidite solutions, 0.1 M

Dry phosphoramidites over anhydrous P_2O_5 for ≥33 hr. Dilute in anhydrous acetonitrile to make each 0.1 M solution (see Table 4.4.3). Use within a week.

Sodium phosphate buffer, 0.5 M, pH 6.8

4.73 g NaH_2PO_4
2.24 g Na_2HPO_4
H_2O to 100 mL

Terminal deoxyribonucleotide transferase (TNT) buffer, 5×

500 mM potassium cacodylate
1 mM dithiothreitol
10 mM $CoCl_2$
Adjust pH to 7.2

COMMENTARY

Background Information

The 2-5A system (Johnston and Torrence, 1984; Player and Torrence, 1998) has been the basis of a targeted mRNA destruction method that derives from the covalent linkage of a (3′-5′)-antisense oligodeoxyribonucleotide and a (2′-5′)-oligoadenylate activator of RNase L, the 2-5A-dependent RNase (Lesiak et al., 1993; Torrence et al., 1993). This composite nucleic acid (Fig. 4.4.1; Xiao et al., 1996) could, through the antisense domain, target the chimera to a particular mRNA sequence, which

Table 4.4.3 Preparation of Automated Synthesizer Reagents

CE phosphoramidite	CH_3CN (mL)	Molarity (M)	Coupling cycles	Sources
500 mg dA^{Bz}	5.6	0.1	25	PE Biosystems
500 mg $dG^{i\text{-}Bu}$	5.8	0.1	24	PE Biosystems
500 mg dC^{Bz}	5.9	0.1	24	PE Biosystems
500 mg T	6.6	0.1	27	PE Biosystems
0.25 g butanediol linker	3.75	0.1	10	Support Protocol 2
500 mg 2-5A	5.0	0.1	24	Support Protocol 1
0.25 g phosphorylation reagent	3.8	0.1	7	Glen Research

would then be targeted for destruction by the 2-5A component via a localized activation of the latent 2-5A-dependent RNase.

The prototype 2-5A-antisense chimera consisted of an antisense domain made up of oligo$(dT)_{18}$ connected to 2-5A through a linker (Lesiak et al., 1993; Torrence et al., 1993). The 2-5A and antisense moieties were joined together through phosphodiester bonds and two 1,4-butanediol molecules linked to each other by a phosphodiester function. Linkage to the 2-5A tetramer was at the 2′-terminal hydroxyl, and linkage to the antisense oligonucleotide was at the 5′-terminal hydroxyl. The mode of linkage to the 2-5A component was through the 2′ terminus of the oligomer, since a free 5′-monophosphate was required for maximal 2-5A-dependent endonuclease activity (Johnston and Torrence, 1984; Player and Torrence, 1998). Linker elements were used to join 2-5A to the antisense DNA sequence rather than directly joining the terminal 2-5A adenosine nucleotide to the first nucleotide of the antisense sequence. Such a strategy was used because of the possibility that RNase L, once bound to the 2-5A component of the chimera, might disturb hybridization to target RNA, or conversely that the double helix generated by the antisense oligonucleotide and the sense RNA might interfere with binding to RNase L.

The 2-5A antisense approach has led to sequence-specific cleavage of a modified human immunodeficiency virus RNA (Torrence et al., 1993) and of mRNA encoding the dsRNA-dependent protein kinase (PKR) in cell-free systems (Maitra et al., 1995), as well as ablation in intact HeLa cells of PKR mRNA, PKR protein, and the biological function of PKR (Maran et al., 1994). In addition, respiratory syncytial virus (RSV) replication has been inhibited by specific 2-5A-antisense targeting of the RSV M2 mRNA (Cirino et al., 1997).

The actual chemical synthesis of 2-5A-antisense oligonucleotides involves the solid-support phosphite-triester approach to DNA/RNA synthesis (e.g., Beaucage and Caruthers, 1981). Appropriately protected 2-cyanoethylphosphoramidite derivatives of the riboadenosine, butanediol linker, and the usual four deoxyribonucleosides are used for chain elongation. Sugar protection consists of the usual 4,4′-dimethoxytrityl for 5′ hydroxyls and *tert*-butyldimethylsilyl for 2′ or 3′ hydroxyls. For base protection, A^{Bz}, C^{Bz}, and $G^{i\text{-}Bu}$ have been used by this laboratory for some time with good success; however, the authors have used commercially available A^{PAC}, $C^{i\text{-}Bu}$, and G^{PAC}, which allow faster ammoniacal workup and yield slightly cleaner crude product. 2-Cyanoethylphosphoramidites are the functionality of choice for phosphodiester bond generation.

The following generic oligonucleotide structural types are described in this unit:

I: 5′-pA(2′,5′-pA)$_3$-2′-p[$(CH_2)_4$p]$_2$dN(3′,5′-pdN)$_n$

II: 5′-pA(2′,5′-pA)$_3$-2′-p[$(CH_2)_4$p]$_2$dN(3′,5′-pdN)$_m$(3′,3′-pdN)

Analytical results are somewhat different when the snake venom phosphodiesterase protocol is applied to a type II (Li et al., 1997) 2-5A-antisense chimera. Specifically, a (3′-3′)-dinucleotide 5′-monophosphate derived from the last two nucleotides of the chimera's 3′ terminus is produced. Structural information to corroborate the identity of the dinucleotide can be obtained by comparing their HPLC on-line UV spectra with the calculated spectra of the 1:1 summation of constituent mononucleotides. Finally, the structure of the (3′-3′)-dinucleotide fragment can be corroborated by comparing the HPLC chromatogram of the enzymatically digested product with that of a synthetic dinucleotide produced by a DNA synthesizer using a nucleoside bound to CPG through the 5′-hydroxyl group.

The retention times of the (3′-3′)-dinucleotide products vary depending upon their composition and, in some cases, can interfere with other peaks in the HPLC analysis of snake venom phosphodiesterase digests. Under these conditions, accurate analysis of these key digestion products can be problematic. In order to obviate this difficulty, digestion with snake venom phosphodiesterase can be carried out with the addition of bacterial alkaline phosphatase. This results in the removal of all non-internucleotide phosphates, so that digestion products consist of nucleosides and the (3′-3′)-dinucleotide. Thus, this modified digestion procedure can result in a shift in retention times (R_t) such that each individual product is well separated from the others. This additional digestion procedure is not always necessary; its necessity depends on the composition of the dinucleotide product. Many (3′-3′)-dinucleotides can be resolved very well from all nucleotides, and can be easily identified by their on-line UV spectra and subsequently integrated.

In general, the syntheses follow the strategies developed in Lesiak et al. (1993), Xiao et al. (1996), and Li et al. (1997). Type I 2-5A-antisense chimeras were the first structural type

synthesized (Lesiak et al., 1993; Torrence et al., 1993) and were used with success in cell-free systems and in intact cells (Torrence et al., 1993; Maran et al., 1994; Maitra et al., 1995). Type II 2-5A-antisense chimeras with 3′-terminally inverted phosphodiester bonds have proven to be of greater stability toward exonuclease degradation and have been exploited to block respiratory syncytial virus replication (Cirino et al., 1997).

Critical Parameters

In the prototypical 2-5A antisense chimera, 2-5A and antisense DNA were joined through two linkers of 1,4-butanediol phosphate that arise from the key intermediate 4-*O*-(4,4′-dimethoxytrityl)oxybutyl-1-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite]. The yield of this intermediate is improved significantly when using 2:10:1 (v/v) ethyl acetate/hexane/triethylamine as the flash purification chromatography solvent. Triethylamine in the purification may prevent decomposition of this acid-sensitive compound during exposure to silica gel.

Key considerations in the characterization of 2-5A-antisense chimeras include: (1) that (2′,5′)-phosphodiester bonds in the RNA portion of the chimera are not isomerized to (3′,5′)-phosphodiesters during or after synthesis; (2) that 1,4-butanediol phosphate linker connects 2-5A and antisense DNA in the anticipated position; and (3) that phosphorylation does, in fact, occur at the 5′ terminus in high yield. Approaches to address these questions may be found in Xiao et al. (1996).

Anticipated Results

Under the conditions described herein, the coupling efficiencies for different regions of the 2-5A-antisense molecule should be: antisense DNA, 98% to 99%; butanediol linkers, 97% to 98%; (2′,5′)-RNA, 94% to 98 %; and phosphorylation, 98% to 99%. The average stepwise coupling yield for 2-5A-antisense chimera should be 96% to 98%.

When 2-5A-antisense oligonucleotides are synthesized and purified according to the procedures outlined herein, products of purities approaching 95% are possible. Isolated yields of purified chimeric oligonucleotides of 70 A_{260} units are achievable.

The digestion of a representative 2-5A-antisense chimera with snake venom phosphodiesterase is shown in Figure 4.4.4. This HPLC picture of the snake venom digestion differs when the substrate for digestion contains a (3′-3′)-phosphodiester-linked dinucleotide. When a representative (3′-3′)-tailed chimera, 5′-pA(2′5′-pA)$_3$-2′p(Bup)$_2$-pd[AAT GGG ATC CAT TTT GTC C(3′-3′)C], is digested under standard conditions with snake venom phosphodiesterase, the HPLC of digestion products will reveal seven major peaks with retention times of 10.8, 29.2, 31.1, 32.59, 39.3, 40.2, and 41.5 min. The first five correspond to, respectively, dCMP, dTMP, dGMP, rAMP, and AMP(pBu)$_2$. At 40.2 min, a new product appears that is not observed in a similar digestion of a standard first-generation 2-5A-antisense chimera with no 3′-3′ linkage. Lastly, dAMP is at 41.5 min. The overall ratio of above nucleotidic digestion products is 3:7:4:3:4 for dCMP/dTMP/dGMP/rAMP/dAMP, revealing an underabundance of dCMP from what would be expected from complete digestion. However, the peak with retention time of 40.2 min possessed a UV spectrum that could be generated by 1:1 addition of the spectra of two mononucleotides, 2 × dCMP. Thus, the structure pdC3′p3′dC can be assigned to this new peak. The structure of this product (3′-3′)-dinucleotide can be corroborated by comparison of the HPLC chromatogram of enzymatically digested product with synthetic dinucleotide.

Similar HPLC digestion patterns were obtained when other (3′-3′)-tailed chimeras were digested with snake venom phosphodiesterase. For instance, pA$_4$-[pBu]$_2$-pd[GCC CAC CGG GTC CAC CAT(3′-3′)C] gave the dinucleotide pdT3′p3′dC (R_t = 46.0 min) and pA$_4$-[pBu]$_2$-pd[TGG GAA GCT GTC ACT GTA GAG(3′-3′)C] yielded pdG3′p3′dC (R_t = 44.4 min). The (3′-3′)-dinucleotides can be assigned structures based on a comparison of their UV spectra with the calculated spectra from 1:1 summation of constituent mononucleotides.

The retention times of the (3′-3′)-dinucleotide products varied depending upon their composition. For instance, the retention time of pdC3′p3′dC is close to that of other digestion products such as dAMP (R_t = 41.5 min) or AMP(pBu)$_2$ (R_t = 40.2 min). Under these conditions, accurate analysis of these key digestion products may be problematic. To deal with this difficulty, digestion with snake venom phosphodiesterase can be carried out with the addition of bacterial alkaline phosphatase. This results in the removal of all non-internucleotide phosphates, so that digestion products consist of nucleosides and the (3′-3′)-dinucleotide. Thus, for instance, when the chimera 5′-pA(2′,5′-pA)$_3$-2′-(pBu)$_2$-pd[AAT GGG ATC CAT TTT GTC C(3′-3′)C] is digested with

snake venom phosphodiesterase and bacterial alkaline phosphatase, the following products are obtained: dC (R_t = 25.6 min), dG (R_t = 42.8 min), dT (R_t = 44.8 min), dC3′p3′dC (R_t = 47.8 min), rA (R_t = 50.1 min), dA (R_t = 51.8 min), and A2′pBupBu (R_t = 54.9). Thus, this modified digestion procedure resulted in a shift in R_t values such that each individual product was well separated from the others. This additional digestion procedure is not always necessary, and its use depends on the constitution of the dinucleotide product.

Time Considerations

The following are estimations of the time needed for various stages of the synthesis, purification, and characterization of a chimeric 2-5A-antisense oligonucleotide.

Support Protocol 1 requires 6 days for synthesis of N^6-benzoyl-5′-*O*-dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyladenosine-2′-*N*,*N*-diisopropylphosphoramidite. Support Protocol 2 requires 2 days for synthesis of 4-*O*-(4,4′-dimethoxytrityl)oxybutyl-1-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite.

Basic Protocol 1 requires 6 to 8 hr for automated 2-5A-antisense chimera synthesis on an ABI 391 or 392 synthesizer, and 3 hr to overnight for cleavage and deprotection of the chimera.

Basic Protocol 2 requires 6 hr for purification of the chimera by polystyrene reversed-phase ion-pair HPLC; 6 hr for desalting and concentration of the chimera; 3 hr for cation exchange; 24 to 48 hr for dialysis and sterilization; 12 hr for capillary gel electrophosesis (a large number of samples may be run in this time when using an autosampler); and 4 hr for the Dionex HPLC purity check.

Basic Protocol 3 requires 6 to 8 hr for snake venom digestion and HPLC analysis of three samples.

Basic Protocol 4 requires 4 hr for labeling the chimera with terminal nucleotide transferase; 4 hr for determining the specific activity of the radioactive chimera; 6 to 8 hr for Maxam-Gilbert sequencing; plus the necessary time for autoradiograph exposure.

Literature Cited

Beaucage, S.L. and Caruthers, M.H. 1981. Deoxyribonucleotide phosphoramidites—A new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett.* 22:1859-1862.

Cirino, N.M., Li, G., Xiao, W., Torrence. P.F., and Silverman, R.H. 1997. Targeting RNA for decay in respiratory syncytial virus infected cells with 2′→5′ oligoadenylate antisense. *Proc. Natl. Acad. Sci. U.S.A.* 94:1937-1942.

Johnston, M.I. and Torrence, P.F. 1984. The role of interferon-induced proteins, double-stranded RNA and 2′,5′-oligoadenylate in the interferon-mediated inhibition of viral translation. *In* Interferon: Mechanism of Production and Action (R.M. Freidman, ed.) pp. 189-298. Elsevier/North-Holland, Amsterdam.

Lesiak, K., Khamnei, S., and Torrence, P.F. 1993. 2′,5′-Oligoadenylate:antisense chimeras—Synthesis and properties. *Bioconjugate Chem.* 4:467-472.

Li, G., Xiao, W., and Torrence, P.F. 1997. Synthesis and properties of second generation 2-5A-antisense chimeras with enhanced resistance to exonucleases. *J. Med. Chem.* 40:2959-2966.

Maitra, R.K., Li, G., Xiao, W., Dong, B., Torrence, P., and Silverman, R.H. 1995. Catalytic cleavage of an RNA target by 2-5A antisense and RNase L. *J. Biol. Chem.* 270:15071-15075.

Maran, A., Maitra, R.K., Kumar, A., Dong, B., Xiao, W., Li, G., Williams, B.R.G., Torrence, P.F., and Silverman, R.H. 1994. Blockage of NF-kB signaling by selective ablation of an mRNA target by 2-5A antisense chimeras. *Science* 265:789-792.

Player, M. and Torrence, P.F. 1998. The 2-5A system: Modulation of viral and cellular processes through acceleration of RNA degradation. *Pharmacol. Therapeut.* 78:55-113.

Torrence, P.F., Maitra, R.K., Lesiak, K., Khamnei, S., Zhou, A., and Silverman, R.H. 1993. Targeting RNA for degradation with a (2′-5′)oligoadenylate-antisense chimera. *Proc. Natl. Acad. Sci. U.S.A.* 90:1300-1304.

Xiao, W., Player, M.R., Li, G., Zhang, K., Lesiak, K., and Torrence, P.F. 1996. Synthesis and characterization of composite nucleic acids containing 2′,5′-oligoriboadenylate linked to antisense DNA. *Antisense Nucleic Acid Drug Devel.* 6:247-258.

Contributed by Mark R. Player and Paul F. Torrence
Northern Arizona University
Flagstaff, Arizona