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# Deoxyribonucleic Acids and Related Compounds. XII.[1] Polymer Support Synthesis of a 46-mer Duplex Containing the Promoter of Galactose Operon of *Escherichia coli*

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A deoxyribonucleic acid (DNA) duplex with a chain length of 46 which contain binding sites for catabolite gene activator protein and ribonucleic acid (RNA) polymerase was synthesized by the solid-phase phosphotriester method, involving condensation of tetramer or pentamer blocks. Eleven oligonucleotide blocks were used to elongate each chain, beginning from polystyrene-linked N-protected 3′-succinyldeoxynucleoside. The estimated overall yields of upper and lower chains were 18% and 17%, respectively. The products were purified by reversed phase chromatography and characterized by sequence analysis using the Maxam–Gilbert method.

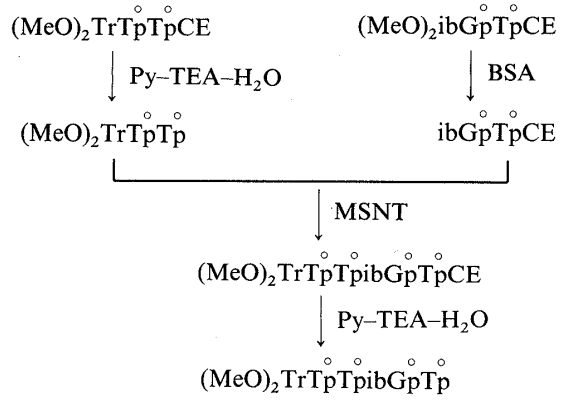
**Keywords**——phosphotriester method; galactose operon promoter; reversed phase chromatography; CD; melting temperature; transcription

Studies on the recognition of deoxyribonucleic acid (DNA) structures by proteins are important to achieve an understanding of the mechanisms for gene expression. Interactions of regulatory proteins and DNA have been investigated in several cases.[2] The galactose operon of *E. coli* was found to contain a promoter site close to the binding regions for catabolite gene activator protein (CAP).[3] We have previously reported syntheses of 15-mer[4] and 22-mer[5] DNA duplexes which corresponded to the ribonucleic acid (RNA) polymerase binding site and the CAP binding site in *E. coli*, respectively, by the phosphoro-*p*-anisidate method in solution. In this paper, we describe a synthesis of a DNA duplex with a chain length of 46 (46-mer), which includes the promoter and CAP sites (Fig. 1). For the synthesis of this relatively large DNA fragment, the phosphotriester solid phase approach was employed with the use of tetra- and pentanucleotide blocks to improve the purity of the product. Preliminary results on the physical properties and transcription of the 46-mer duplex are also reported.

## Synthesis of Tetra- and Pentanucleotide Blocks

Mathods for phosphotriester synthesis on a polymer support have been improved recently, as discussed in a review article.[6] A large scale synthesis of deoxyribooligonucleotides on a polystyrene support by the phosphotriester approach has been reported.[7] However, the solid phase synthesis involving multi-step condensations requires extensive purification of final products. To reduce the number of reaction steps, larger oligonucleotide blocks are suitable intermediates, although purification of tetra- or pentanucleotide blocks on silica gel or alkylated silica gel is time-consuming. In fact, the use of tetranucleotide blocks gave better yields as compared to those for dimers in the synthesis of a gene fragment with a chain length of 25 nucleotides.[8] Preparation of completely purified tetra- or pentanucleotides and their use in solid-phase synthesis seemed to be a promising method for the rapid synthesis of a 46-mer oligonucleotide.

Altogether, 20 tetranucleotides and two pentanucleotides were synthesized from dinucleotides[9] according to the procedure shown in Chart 1 for the synthesis of protected




p: *o*-chlorophenyl phosphoryl
$(MeO)_2Tr$: dimethoxytrityl
CE: *β*-cyanoethyl
ibG: *N*-isobutyryldeoxyguanosine
MSNT: 1-(mesitylenesulfonyl)-3-nitrotriazole
BSA: benzenesulfonic acid
TEA: triethylamine

Chart 1. Synthesis of a Tetranucleotide Block

d(TTGT)p. The fully protected tetranucleotides were separated by chromatography on silica gel. The 3′-monoesterified tetranucleotides could be further purified by reversed phase chromatography. The yields of the protected tetranucleotide blocks were 64—100%.

**Synthesis of the 46-mers on a Polymer Support**

Aminomethylated polystyrene with 1% cross-linkage was used as the support. 3′-*O*-Succinyl-*N*-benzoyl deoxycytidine was linked to the polymer for the synthesis of the upper strand (Fig. 1) and 3′-*O*-succinyl-*N*-isobutyryldeoxyguanosine was joined to the support for the lower strand, *via* pentachlorophenyl esters.[10] Ten tetramers were condensed by using 1-(mesitylenesulfonyl) 3-nitro-1,2,4-triazole (MSNT)[11] as the activating reagent. Each reaction

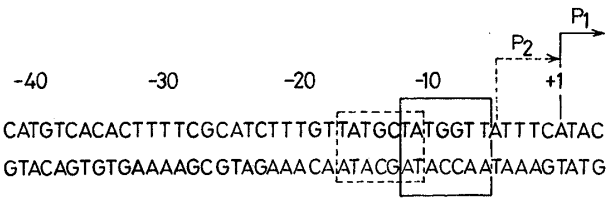



Fig. 1. The Promoter Sites of the *gal* Operon of *E. coli*

The symbol +1 indicates the initiation site in the presence of cyclic adenosine monophosphate (cAMP) and CAP when RNA polymerase binds to the putative promoter regions (solid lines). P2 indicates the initiation site in the absence of CAP, and promoter regions are marked by dotted lines.

TABLE I. Synthetic Cycle

| Step | Solv or Reagent | Volume (ml) | Time (min) | Repetition |
|---|---|---|---|---|
| 1 Wash | $CH_2Cl_2$–MeOH (7:3 v/v) | 2 | 0.1 | 3 |
| 2 Detritylation | 2% BSA | 2 | 1 | 1 |
| 3 Wash | $CH_2Cl_2$–MeOH (7:3 v/v) | 2 | 0.1 | 1 |
| 4 Detritylation | 2% BSA | 2 | 1 | 1 |
| 5 Wash | $CH_2Cl_2$–MeOH (7:3 v/v) | 2 | 0.1 | 3 |
| 6 Wash | Pyridine | 2 | 0.1 | 3 |
| 7 Drying | Nucleotide block + resin, pyridine (0.3 ml) co-evaporation | | | |
| 8 Coupling | MSNT, super-pyridine (0.3 ml), 40 °C, 20 min | | | |
| 9 Wash | Pyridine | 2 | 0.1 | 2 |
| 10 Acetylation | $Ac_2O$ | 0.2 | | |
| | 0.1 M DMAP in pyridine | 1.8 | 3 | 1 |
| 11 Wash | Pyridine | 2 | 0.1 | 2 |

DMAP, dimethylaminopyridine.

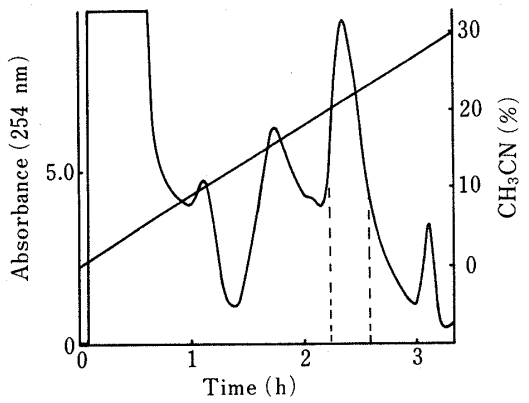



Fig. 2a. Reversed-Phase Chromatography of the Dimethoxytrityl 46-mer (Upper Strand)

C-18 silica gel (35—105 $\mu$) was packed in a column (0.9 × 9 cm). Elution was performed with a gradient of acetonitrile (2—30%, total 200 ml) in 0.1 M ammonium acetate.

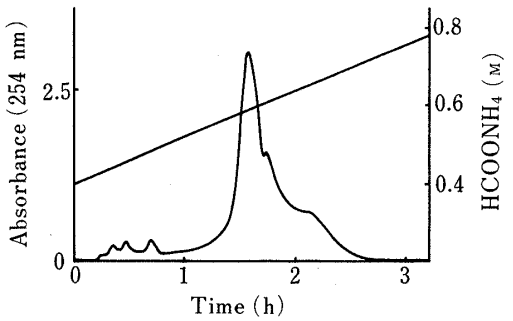



Fig. 2b. Anion-Exchange Chromatography of the Deblocked Upper Strand (Obtained in the Fractions Shown in Fig. 2a) on a Column (0.8 × 20 cm) of Toyopearl 650S

Elution was performed with a gradient of ammonium formate (0.4—1.0 M) in 20% acetonitrile.

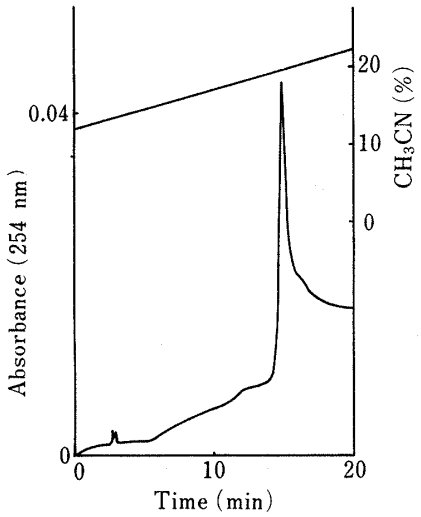



Fig. 3a. Reversed-Phase HPLC of the Upper Strand after the Anion-Exchange Chromatography Shown in Fig. 2b

A column (4 × 250 mm) of C-18 silica gel (TSK-410AK) was used. The flow rate was 1 ml/min.

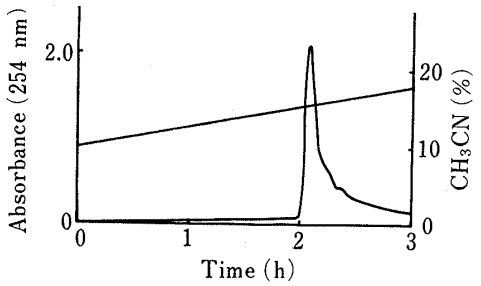



Fig. 3b. Reversed-Phase Chromatography of the Lower 46-mer in a Column (0.9 × 13.5 cm) of TSK-Gel LS-410 ODS-SIL (10—20 $\mu$)

Elution was performed with a gradient of acetonitrile (7—18%) in 0.1 M ammonium acetate. The total volume was 200 ml.

cycle involved eleven operations as summarized in Table I. The pentanucleotide was reacted at the last step. Yields were estimated by utilizing the color reaction of dimethoxytritanol[10] removed from the elongating chain except for the last step, where an aliquot of the product linked to the support was used for the estimation. The average yield for the upper strand was 86% and the overall yield was 8%. The lower strand was synthesized by a similar procedure. The oligonucleotide was removed from the support and partially deblocked by treatment with 1,1,3,3-tetramethylguanidinium *syn*-pyridine-2-carboaldoximate[11] followed by ammonia treatment. The dimethoxytritylated oligonucleotide was isolated by reversed-phase chromatography (Fig. 2a). The dimethoxytrityl group was removed with 80% acetic acid and the completely deblocked product was purified by ion-exchange chromatography using ammonium formate as an elution buffer (Fig. 2b). The desalted product was further purified by high-performance liquid chromatography (HPLC) on C-18 silica gel (Fig. 3a) or by using C-

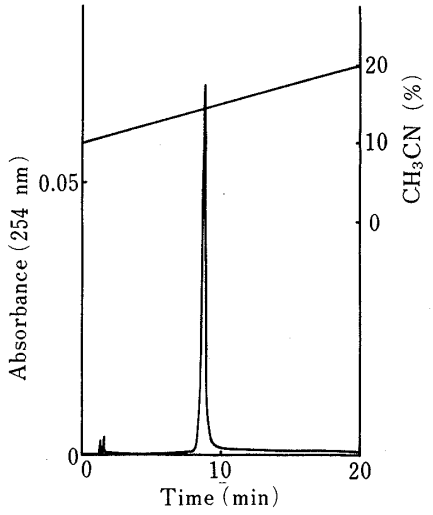



Fig. 4. Analytical HPLC of the Lower Strand Isolated as Shown in Fig. 3b on a Column (4 × 15 mm) of C-18 Silica Gel (Accupack)

The flow rate was 1 ml/min.

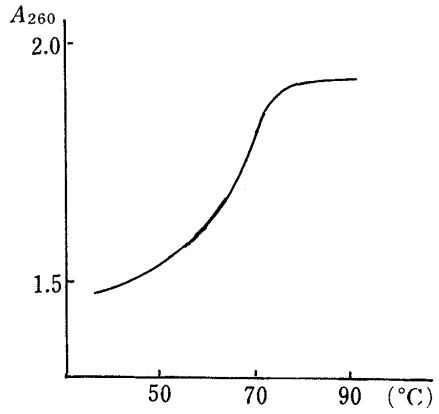



Fig. 5. UV–Temperature Profile for the 46-mer Duplex

Each strand (0.8 $A_{260}$ unit) was dissolved in 0.1 M NaCl in the presence of 10 mM sodium phosphate (pH 7.0) and the solutions were mixed. The temperature was raised at 0.5 °C/min.

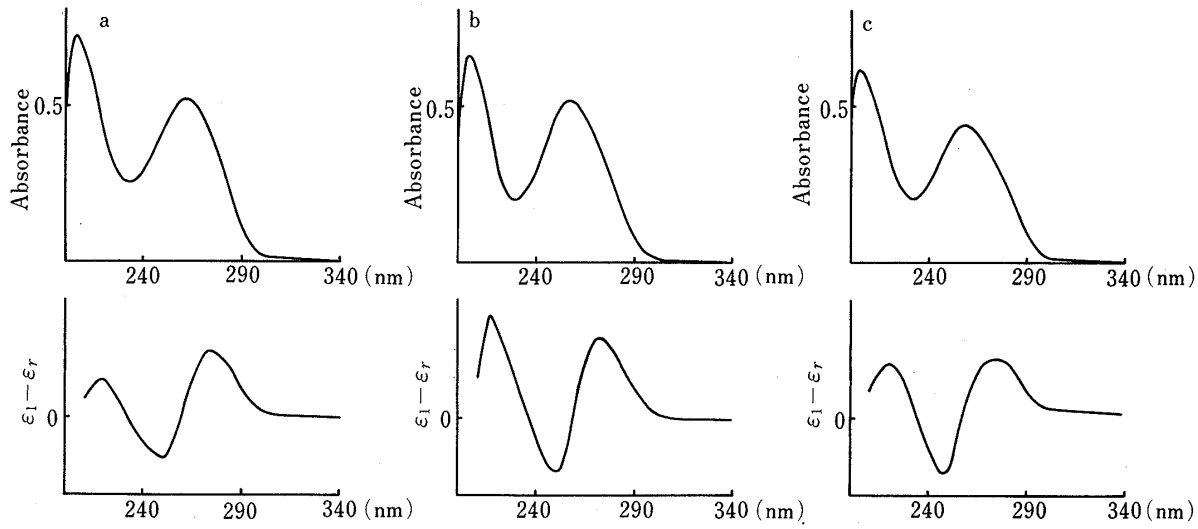



Fig. 6. UV and CD Spectra for the Upper Strand (a), the Lower Strand (b) and the Duplex (c) in 0.01 M Sodium Phosphate (pH 7) in the Presence of 0.1 M Sodium Chloride

18 silica gel (coarse) at medium pressure (Fig. 3b). The purity of the products was finally checked by analytical HPLC and polyacrylamide gel electrophoresis.[12] Figure 4 shows the result of reversed-phase HPLC of the 46-mer isolated by the chromatography shown in Fig. 3b. The 5′-end was analyzed by digestion with nuclease P1 after labeling as described previously,[13] and the base sequences were analyzed by the Maxam–Gilbert method.[14]

**Properties of the 46-mer Duplex**

The ultraviolet (UV) absorption–temperature profile for the 46-mer duplex was observed in the presence of 0.1 M salt and the $T_m$ was found to be 66.8 °C as shown in Fig. 5.

Circular dichroism spectra (CD) of the 46-mers were measured under conditions similar to those of the above experiment. The spectra for the two independent 46-mers differed only at the 220 nm region where the lower strand had a larger $\theta$ value than the other strand. The

CD spectra of the two 46-mers together with the UV spectra are shown in Fig. 6a, b. The spectrum of the duplex (Fig. 6c) showed a shape typical of B-form DNA with right-handed helicity. This may mean that DNA regions corresponding to the promoter or CAP binding site are recognized by RNA polymerase or CAP by the interaction of amino acid residues with specific sites of DNA, instead of recognition of a unique shape.

The recognition of this 46-mer duplex by RNA polymerase was studied. Experiments on the transcription with RNA polymerase of the 46-mer duplex showed that the termini of this short duplex might be unstable, since analyses of the transcription products indicated heterogeneous initiation. Instead of yielding pppAUUUCAUAC, which was the presumed initiation product from $P_2$ (Fig. 1) in the absence of guanosine triphosphate (GTP), oligomers (2—9 bases) were detected in polyacrylamide gel electrophoresis. Control experiments using DNA with a chain length of 205 containing the *lac* promoter at the 63 position from the 3′-end gave a tetramer in the absence of GTP and larger fragments in the presence of the 4 nucleotides.

The effect of CAP in transcription with RNA was also tested by a similar procedure. However, no marked difference was observed on addition of CAP. It seems that for specific interaction with proteins, DNA duplexes have to keep a stable form.

## Conclusion

By using tetra- or pentanucleotide blocks, deoxypolynucleotides with a chain length of 46 were synthesized by the phosphotriester solid-phase method. Preparation of protected tetranucleotides was performed in solution and isolation of the product by column chromatography on silica gel was a time consuming procedure. However, relatively large oligonucleotide blocks (*e.g.* tetra- or pentanucleotides) are useful intermediates for the synthesis of polynucleotides longer than 20, since the isolation procedures can be simplified because the smaller number of condensing steps yields a purer product.

For transcription with RNA polymerase it seems necessary to prepare a larger DNA duplex to observe specific interactions. By using DNA ligase, protruding deoxyoligonucleotides can be elongated to form larger duplexes. Combinations of organic and biochemical methods to obtain DNA duplexes with chain lengths of larger than 100 should be useful not only to synthesize structural genes but also to synthesize substrates for many biological reactions.

## Experimental

Thin layer chromatography (TLC) was performed on plates of silica gel (Kiesel gel 60 $F_{254}$, Merck) with a mixture of chloroform and methanol. For reversed-phase TLC (RTLC), silanized silica gel or HPTLC RP-18 (Merck) was used. For column chromatography, Silica gel 60H (Merck), alkylated silica gel (C-18, 35—105 $\mu$, Waters, YMC-Gel C-18 30—50 $\mu$, Yamamura Chemical or TSK-Gel LS410 ODS-SIL, 10—20 $\mu$, Toyo Soda) and anion-exchanger (DEAE-Toyopearl 650 S, Toyo Soda) were used. For HPLC, C-18 silica gel (TSK-Gel LS-410 AK or Accupack) and ion-exchanger (TSK-Gel IEX 540 K) were used.

Other general methods for the preparation and characterization of oligonucleotides were as described previously.[4,5] UV and CD spectra were measured with a Hitachi model 200-10 instrument, a UVIDEC 610C double-beam spectrophotometer (JASCO), DU-8B Spectrophotometer (Beckman) or a J 500 A spectrophotometer (JASCO).

RNA polymerase and CAP from *E. coli* were provided by Dr. A. Ishihama, Kyoto University.

**Removal of the Cyanoethyl Group**——For example, 5′-*O*-dimethoxytritylthymidylyl-(3′-5′)-(*o*-chlorophenyl)-thymidylyl-3′-$\beta$-cyanoethyl(*o*-chlorophenyl)phosphate (235 mg, 0.195 mmol) was treated with pyridine–triethylamine–$H_2O$ (3 : 1 : 1, 2.7 ml) at room temperature for 10 min. The mixture was checked by TLC and concentrated to yield a caramel. For storage, the nucleotide was dried by evaporation with pyridine twice, then dissolved in dichloromethane (5 ml) and precipitated with a mixture of *n*-hexane (50 ml), ether (20 ml) and triethylamine (0.2 ml). The precipitate was washed with the same mixture three times and dried over $P_2O_5$ *in vacuo*.

**d(MeO)$_2$TrTp̊Tp̊ibGp̊Tp̊CE**——d(MeO)$_2$TribGp̊Tp̊CE (195 mg, 0.15 mmol) was dissolved in dichloromethane

(2.3 ml), and benzenesulfonic acid (0.45 mmol) in methanol (1.2 ml) was added at 0 °C. Removal of the dimethoxytrityl group was confirmed by TLC after 30 min. Saturated $NaHCO_3$ (2 ml) was added to the mixture. The washed organic layers were concentrated with pyridine, coevaporated twice and combined with $d(MeO)_2T\overset{\circ}{p}T\overset{\circ}{p}$ (0.19 mmol). The mixture was dried by evaporation with pyridine and treated with MSNT (0.4 mmol) in pyridine (1.2 mmol) for 45 min at room temperature. Completion of the reaction was confirmed by RTLC and HPTLC. The product was extracted with dichloromethane twice after addition of aqueous pyridine (50%, 1 ml). The organic layer was washed twice with water, concentrated with pyridine and applied to a column of Silica gel 60H (7 g). The product was eluted with a gradient of methanol (1—6%) in dichloromethane containing 0.1% pyridine (1.2 l). A homogeneous fraction was obtained in a yield of 97% (310 mg, 0.14 mmol).

**Synthesis and Purification of the 46-mers**——Upper Strand: *N*-Benzoyldeoxycytidine resin (1% cross-linked, 50 mg, 6 $\mu$mol) was treated according to the procedure shown in Table I. Oligonucleotides (42 mg, 18 $\mu$mol) and MSNT (36 mg, 121 $\mu$mol) were used in each condensation at 20 °C for 45 min. For deblocking, the resin was washed 5 times with aqueous dioxane (90%, 2 ml each), shaken with 1 M tetramethylguanidinium-pyridine aldoxime (TMG-PAO) (90%, 1.25 ml) for 41 h at 20 °C and washed 4 times with aqueous pyridine (5 ml each). The combined solutions were dried, dissolved in pyridine (0.5 ml) and treated with conc. ammonia (10 ml) for 4.5 h at 55 °C. The mixture was concentrated and the residue was dissolved in 0.1 M triethylammonium acetate (1.0 ml). An aliquot (10 $\mu$l) was used for estimation of the yield.[10] The crude product (1.17 $\mu$mol) was applied to a column of C-18 silica gel as shown in Fig. 2a and fractions (41—58) were rechromatographed under the same conditions. The main part was dried and treated with 80% acetic acid (2.0 ml) for 20 min at 20 °C. The detritylation was confirmed by TLC and the product was chromatographed on DEAE-Toyopearl 650 S using ammonium formate in the presence of acetonitrile as shown in Fig. 2b. Fractions (25—26) were desalted by gel filtration on Sephadex G-25 and checked by reversed phase HPLC. The product was further purified by chromatography on C-18 silica gel either by using either YMC-Gel 3—30/50 or TSK-Gel LS 410 ODS-Sil (10—20 $\mu$) (for precolumns) under medium pressure, the same conditions as used for the lower strand (Fig. 3b). The purity of the product was tested by analytical HPLC and 10% polyacrylamide gel electrophoresis. The base sequence was analyzed by the Maxam–Gilbert method.[14]

Lower Strand: The lower strand was prepared by essentially the same procedure as above except that *N*-isobutyryldeoxyguanosine resin (126 mg, 12 $\mu$mol), oligomer blocks (70 mg, 30 $\mu$mol) and MSNT (60 mg, 202 $\mu$mol) were used in the condensing reactions at 35 °C for 30 min. The acetylation was performed at 20 °C for 5 min. The dimethoxytrityl 46-mer (1.38 $\mu$mol) was obtained and the 46-mer was finally purified as shown in Fig. 3.

**Transcription of the 46-mer Duplex**——The 46-mer duplex (3 pmol) was used in reactions with nucleoside triphosphates (1 $\mu$mol each), $\gamma$-$^{32}$P adenosine triphosphate (250 pmol) and RNA polymerase (35 pmol) in 50 mM Tris–HCl, 50 mM NaCl, 0.1 mg/ml bovine serum albumin at pH 7.6 (total 7.5 $\mu$l). The reaction was carried out for 15 min at 30 °C and stopped by heating with a mixture (70% formamide, 20% glycerol, 0.2% sodium dodecyl sulfate, bromophenol blue, xylene cyanol) (10 $\mu$l) for 1 min at 75 °C. The whole was subjected to 25% polyacrylamide gel electrophoresis at 200—500 V. Radioactivity was detected by autography. Products were eluted and analyzed by mobility shift analysis[15] as described previously.[12]

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