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Simultaneous rapid chemical synthesis of over one hundred oligonucleotides on a microscale

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An inexpensive, extremely rapid manual method for simultaneous synthesis of large numbers of oligodeoxyribonucleotides on 50 or 150 nanomole scale is described. The oligonucleotides are assembled in parallel by the phosphotriester method on small cellulose paper disks in a simple gas pressure-controlled continuous-flow system. For each addition of a nucleotide the disks are sorted into four sets which are placed in four columns for addition of A, C, G and T, respectively. During one 2-week period, three rounds of synthesis by this method yielded 254 oligomers (8- to 22-mers), many of which were also purified during this time. Using 50 nanomole scale reactions the yields for 17-mers, for example, were in the range of 0.5 O.D.$_{260}$ units (~5 nmol, i.e., ~10% yield), an amount sufficient for most purposes. The equipment required is relatively inexpensive and for the most part usually already available in molecular biology laboratories. All chemicals are commercially available and the current reagent cost per oligonucleotide (25 μg, average length 17-mer) is ~3 US dollars.

Key words: gene synthesis/oligonucleotide purification/oligonucleotide synthesis/phosphotriester method/solid-phase DNA synthesis

Introduction

The cost, in terms of time and materials, has been the most limiting factor in oligonucleotide synthesis. One of the major cost problems has been the inability to scale down on precious reagents without affecting yields. For this reason, currently used synthesis protocols start with at least 1 – 5 μmol of nucleoside attached to the solid support. They then result in quantities of oligonucleotides which are excessive for most purposes, and they lead to a price which is too high for general use of oligonucleotides in large numbers.

Crea and Horn (1980) have described the use of cellulose as a solid support for the triester method. More recently, Frank *et al.* (1983) published a method for simultaneous synthesis of oligonucleotides on cellulose paper support. Here we describe a microscale method using this support and a continuous-flow system. This method makes parallel synthesis of large numbers of oligonucleotides possible, minimising labour and reagent costs.

Results

We have used the microscale method described here for the synthesis of 254 different oligodeoxyribonucleotides, whose length varied from eight to 22 nucleotides. These oligonucleotides have been synthesised in three batches, each batch comprising 60 – 125 different oligonucleotides. In one synthesis of 125 oligomers, the assemblies were carried out in 3 days including binding of the first nucleoside to the support. The majority of this time was spent sorting the disks for each addition cycle.

The phosphotriester method was used mainly as described by Gait *et al.* (1982a, 1982b). By means of a catalyst (Efimov *et al.*, 1982; Sproat and Bannwarth, 1983), 1-methylimidazole, we were able to cut down the coupling times to 15 min, which shortened the total cycle time (except for sorting of the disks) to 27 min. This catalyst was also used in the couplings of the protected nucleoside succinates to the cellulose supports. In this way we could use less reagents and decrease the time of the reaction from an overnight treatment to 1 h. Furthermore, we obtained higher loadings than previously reported for paper support (Frank *et al.*, 1983). Using these modifications, 0.03 – 0.10 μmol of nucleoside were bound per disk of Whatman grade 1 Chr paper and 0.10 – 0.30 μmol per disk of 3 MM paper, which corresponds to 15 – 50 μmol/g. When the treatment was prolonged to 2 – 3 h, the loadings reached 80 μmol/g. In the detritylation steps during the assemblies, dichloroacetic acid (3% in 1,2-dichloroethane) was used (Adams *et al.*, 1983), because it is a more stable and workable reagent than the previously used trichloroacetic acid.

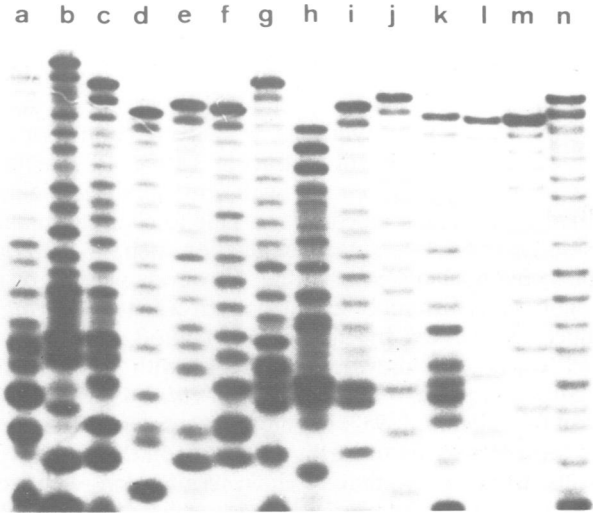

Fig. 1. Autoradiogram of the crude mixtures of 14 different 14- to 18-mers (**a** – **n**) after ^{32}P 5′ end-labelling and gel electrophoresis (20% polyacrylamide, 8 M urea). The sequences of the oligonucleotides are as follows: **a**, 5′-CTTCTGAGGCGGAAAGA-3′; **b**, 5′-ACCAGCTGTGGAATGTGT-3′; **c**, 5′-GTCAGTTAGGGTGTGG-3′; **d**, 5′-AAAGTCCCCAGGCTCC-3′; **e**, 5′-CCAGCAGGCAGAAGTA-3′; **f**, 5′-TGCAAAGCATGCATCT-3′; **g**, 5′-CAATTAGTCAGCAACCAG-3′; **h**, 5′-GATCCTGGTTGCTG-3′; **i**, 5′-ACTAATTGAGATGCAT-3′; **j**, 5′-GCTTTGCATACTTCTGC-3′; **k**, 5′-CTGCTGGGGAGCCTG-3′; **l**, 5′-GGGACTTTCCACACCC-3′; **m**, 5′-TAACTGACACACATTC-3′; **n**, 5′-CACAGCTGGTTCTTTCC-3′.

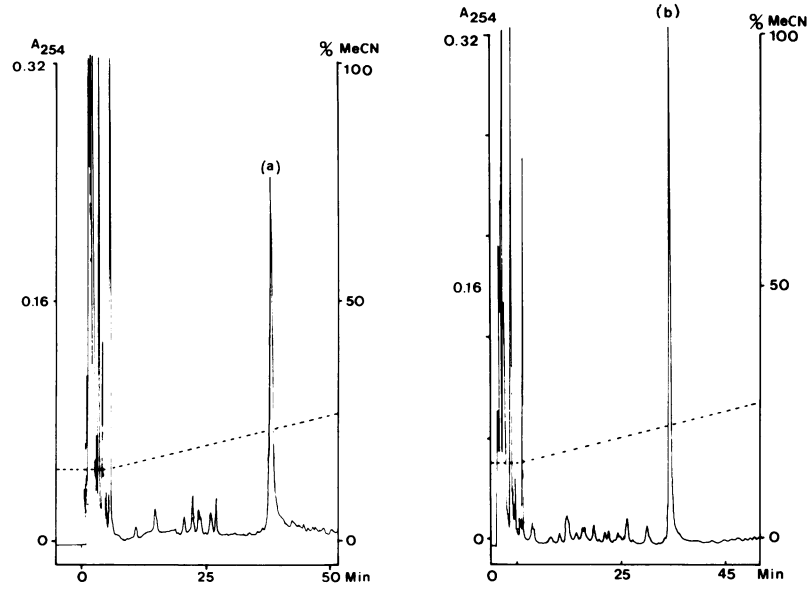

Fig. 2. Purification of 5′-O-DMT-oligonucleotides by reverse phase h.p.l.c. Elution profile of: **(a)** 5′-O-DMT-CAATTAGTCAGCAACCAG-3′, 18-mer; **(b)** 5′-O-DMT-GCTTTGCATACTTCTGC-3′, 17-mer.

The yields for 17-mers, for example, on papers initially loaded with 50 nmol, varied from 0.1 to 1 O.D.$_{260}$ units of pure product. This is approximately a 2–20% yield compared with loading, with an average yield of ~10% pure product, corresponding to an 87% yield per cycle. Thus, in spite of the fact that we opened up the system after each coupling, our assembly efficiencies are comparable with those obtained previously on beaded supports (Gait *et al.*, 1982a; Efimov *et al.*, 1982; Sproat and Bannwarth, 1983).

To analyse the reaction products immediately after synthesis and deprotection, aliquots of various detritylated oligonucleotides were 5′ end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase and subjected to gel electrophoresis under denaturing conditions. Figure 1 shows the autoradiogram of an analysis of 14 different 14- to 18-mers. In all the cases the longest oligonucleotide, corresponding to the desired product of the synthesis, is a strong band. For most preparations the full length product is ~10% of the material, which is in good agreement with the obtained yields of purified products discussed above. Due to chain termination by side reactions after each elongation step, a pattern of smaller oligonucleotides is visible. However, we do not find any significant difference in this pattern when comparing the products synthesised by the present method with those obtained using our previous protocols with polydimethylacrylamide-kieselguhr resin (Gait *et al.*, 1982a, 1982b), Porasil (Kohli *et al.*, 1982) or, more recently, controlled pore glass (Sproat and Bannwarth, 1983) as solid supports (data not shown). Thus, premature chain termination seems to be more dependent on impurities in the reagents than on the type of support used. In Figure 1 it can be seen that the majority of non-complete oligomers are short (length 2–5 nucleotides), indicating that more chain terminations occur in the first steps of the assemblies than in later cycles. This shows that there may be a steric hindrance on the support which either allows side reactions, especially during the first additions, or which simply hinders the chain from growing any further. This hindrance could perhaps be decreased by the addition of a spacer group to the cellulose support.

Purification by reverse phase h.p.l.c. gave, in almost all cases, the 5′-O-dimethoxytrityl-oligonucleotide (5′-O-DMT-oligonucleotide) as a single sharp peak, sometimes followed by a few small peaks of shorter oligonucleotides. Figure 2 shows the elution profiles from two typical reverse phase h.p.l.c. purifications. The elution time varied between 32 and 45 min with an average elution time of 38 min. The retention of the 5′-O-DMT-oligonucleotides is sequence and length dependent, with longer oligomers eluting earlier since the effect of the hydrophobic 5′-O-DMT-protecting group diminishes with the length.

Linkers and adaptors were best purified by preparative gel electrophoresis, since they can self-hybridise and give rise to additional peaks under normal reverse phase h.p.l.c. conditions. Figure 3A and B shows 10 and seven u.v. shadowings respectively, of preparative oligonucleotide purifications on polyacrylamide gels. Figure 3A shows that the detritylated synthesis mixtures can be purified on polyacrylamide gels without further pre-treatment. However, as seen in Figure 3B, the band separation was better after ethanol precipitation despite the fact that three times more material was loaded per slot area (see legend to Figure 3). A DEAE column purification of the synthesis mixtures further improved the band separations at high loadings per slot area (data not shown), but is normally not necessary.

We routinely characterised the purified oligonucleotides by polyacrylamide gel electrophoresis after ^{32}P 5′ end-labelling with T4 polynucleotide kinase. Figure 4 represents an autoradiogram of the electrophoretic analysis of 21 different 5′ end-labelled oligonucleotides after h.p.l.c. (Figure 4A) or gel purification (Figure 4B). After either purification method the oligonucleotides had a purity >99%. In the case of an 18-mer (5′-GGCGCTGACGACAGGTGC-3′) the sequence was confirmed by Maxam-Gilbert sequence analysis (see Figure 5). It should be noted that the kinations were as efficient as those of oligonucleotides synthesised by the previously used methods.

After 5′ end phosphorylation with T4 polynucleotide kinase, the synthesised oligonucleotides could be ligated effi-

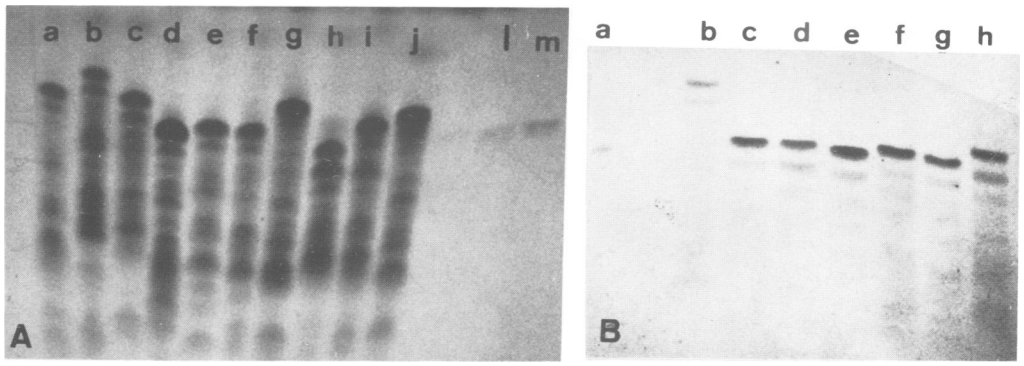

Fig. 3. U.v. shadowing of oligonucleotides separated by polyacrylamide gel electrophoresis. **(A)** Preparative polyacrylamide gel electrophoresis (1 mm thick gel) of 10 different 14- to 18-mers (**lanes a – j**, same oligonucleotides as lanes a – j of Figure 1) directly after detritylation without prior purification. One quarter of each synthesis (on 150 nmol scale) was loaded per 10 mm^2 slot area. **Lanes l** and **m** are 1 and 2 μg of a 15-mer size marker, respectively. **(B)** Polyacrylamide gel electrophoresis (0.3 mm thick gel) of seven different 15- to 18-mers purified by ethanol precipitation (**lanes b – h**). One eighth of each synthesis (on 150 nmol scale) was loaded per 2 mm^2 slot area. **Lane a** contains 1 μg of a pure 15-mer as a marker; **b**, 5′-ACCAGCTGTGGACTGTGT-3′; **c**, 5′-TAACTGAAACACATTC-3′; **d**, 5′-GGGACTGTCCACACCC-3′; **e**, 5′-GGGAATTTCCACACCC-3′; **f**, 5′-CTGATGGGGAGCCTG-3′; **g**, 5′-CCAGCAGGCAGCCTTA-3′; **h**, 5′-GCTTTGCATAAGGCTG-3′.

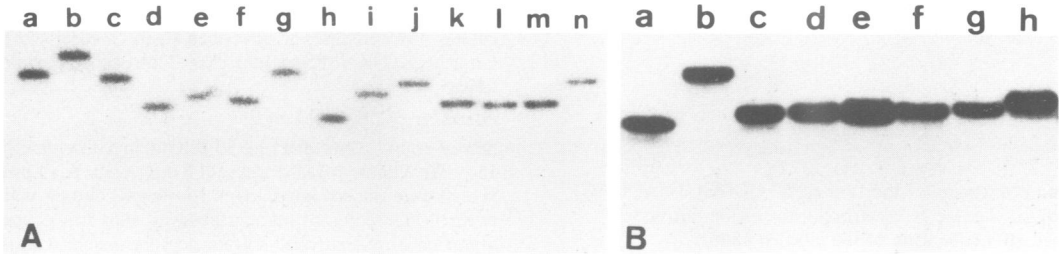

Fig. 4. Autoradiogram of polyacrylamide gel electrophoresis of 21 different purified oligonucleotides after 5′ end-labelling with [γ-^{32}P]ATP and T4 polynucleotide kinase. **(A)** Gel electrophoresis of 14 h.p.l.c. purified 14- to 18-mers. The oligonucleotides in **lanes a – n** are the same as those in lanes a – n of Figure 1. **(B)** Gel electrophoresis of the seven oligonucleotides purified from the polyacrylamide gel shown in Figure 3B. **Lanes b – h** correspond to samples from lanes b – h of Figure 3B. **Lane a** contains an h.p.l.c. purified 15-mer.

ciently. Twelve overlapping 14- to 18-mers were successfully hybridised and ligated with T4 DNA ligase to yield a synthetic DNA fragment of 97 bp (data not shown). Six of the oligonucleotides purified by either of the described methods were successfully used as primers in dideoxy sequencing with the 'Klenow fragment' of *Escherichia coli* DNA polymerase I (Sanger *et al.*, 1977) (data not shown). For example, an M13 sequencing primer, the 17-mer 5′-GTAAAACGACGGCCAGT-3′, was synthesised on a 50 nmol scale in parallel with 59 other oligonucleotides using the present method. We obtained a yield of 0.3 $O.D._{260}$ units which, when used at a rate of 0.2 pmol per M13 sequencing reaction, is sufficient to perform 15 000 such reactions. The oligonucleotide was found to be as efficient as the preparation of Duckworth *et al.* (1981) for priming in dideoxy sequencing reactions (data not shown). Thus, we conclude that oligonucleotides obtained by the present method are biologically active.

Discussion

As synthetic oligonucleotides continue to have more applications in molecular biology there is a growing need to make them available in large numbers to any molecular biologist who wants to use them. The aim of the present study was to develop simple, rapid and inexpensive techniques for the simultaneous synthesis and purification of large numbers of unique oligonucleotides.

The majority of applications requires only picomole quantities of synthetic DNA, whereas the presently available manual or automated DNA synthesisers work on at least a 1 – 5 μmol scale; therefore they require considerable amounts of expensive reagents and solvents and produce a vast excess of material. For example, we recently synthesised a 31-mer on a 1.5 μmol scale using controlled pore glass as solid support, and obtained 30 $O.D._{260}$ units of pure oligonucleotide of which only 15 pmol (0.01%) were ultimately used. In the present study the amounts of chemicals and solvents used to synthesise simultaneously ~100 different oligonucleotides on small paper disks were the same as those previously used for synthesising large amounts of only four oligonucleotides on beaded resins. The assembly is done manually and the rate-limiting step is no longer the coupling reaction itself, but the time needed to sort the paper disks for the subsequent synthesis cycle. The protocol is designed in such a way as to enable every biologist with minimal background in organic chemistry to use commercially available high-purity monomers to synthesise oligonucleotides with a limited amount of work and investment in new equipment.

We preferred the purification of large numbers of oligonucleotides by preparative gel electrophoresis as an alternative to h.p.l.c. An electrophoresis apparatus is available in every molecular biology laboratory, but an h.p.l.c. apparatus remains expensive. Moreover, gel electrophoresis has the

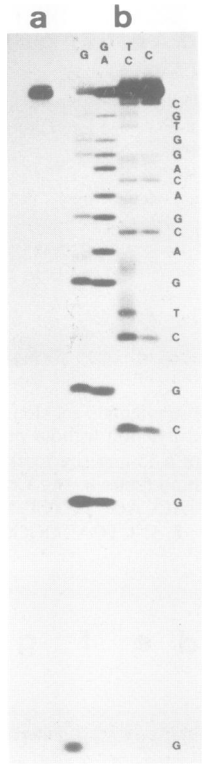

Fig. 5. Maxam-Gilbert sequencing of the 18-mer, 5′-GGCGCTGACGAC-AGGTGC-3′. **(a)** Polyacrylamide gel electrophoresis of the purified ^{32}P 5′ end-labelled 18-mer. **(b)** Sequencing of the labelled 18-mer.

advantage that numerous oligonucleotides can be purified simultaneously, whereas purification by h.p.l.c. works sequentially and requires a minimum of 1 h per oligonucleotide. This is a very relevant point since the oligonucleotides made during 4 days of assembly and deprotection would take ~1 month to purify by h.p.l.c., whereas they could be purified on gels in a few days. Furthermore, the oligonucleotides recovered by preparative gel electrophoresis are pure enough and in amounts sufficient for most applications in molecular biology.

Oligonucleotides synthesised using the procedures described here have been used for different purposes and shown to be active in several different types of enzymatic reactions. Their behaviour is chemically and biologically indistinguishable from that of oligonucleotides prepared by conventional methods. There is a potential source of sequence error in the oligonucleotide syntheses during sorting of the disks before the coupling steps, but by carefully following pre-determined protocols and by double checking at each coupling step we believe that a cautious worker can reduce the probability of such an error to less than one in a thousand. Computer designed and stored protocols may further decrease the frequency of such mistakes. No errors have been found to date in the sequences of the oligonucleotides which have been recently synthesised and used in our laboratory. Their use in different projects is currently in progress and will be reported separately.

Finally, it should be emphasized that the method described in this paper is of special interest for the chemical synthesis of wild-type or mutated genes from overlapping oligonucleotides, since it provides a way of producing efficiently a large number of synthetic oligonucleotides simultaneously in a very short time. Based on 16- to 22-mers, the number of oligonucleotides required to cover a gene of 1–2 kb can be synthesised and purified within 2 weeks by a single person. Clearly our present method will also be extremely useful to synthesise probes for screening cDNA libraries (Suggs *et al.*, 1981; Noda *et al.*, 1983) or genomic libraries (Anderson and Kingston, 1983). We hope that the present paper will encourage molecular biologists to synthesise oligonucleotides themselves.

Materials and methods

The phosphotriester method was used mainly as described earlier (Gait *et al.*, 1982a, 1982b) with some recent improvements (Efimov *et al.*, 1982; Sproat and Bannwarth, 1983). The solid supports were Whatman 1 or 3 MM chromatography (Chr) papers cut to round disks of 6 mm diameter. The glass columns and the solvent delivery system (4-column model with an eight-way valve no. 1103) were from Omnifit, Cambridge (UK). Hamilton 1750 RN 0.5 ml syringes were used. Anhydrous pyridine and 1,2-dichloroethane (DCE, pure), were purchased from SDS, F-13124 Peypin, France, and used as such. Dichloroacetic acid (DCA) was from Fluka. 1-Methylimidazole was from Merck-Schuchardt (No. 805852) and was stored over 4A molecular sieves. 4-Dimethylaminopyridine (DMAP) was purchased from Aldrich. 1-mesitylenesulphonyl-3-nitro-1,2,4-triazolide (MSNT), triethylammonium (TEA) salts of protected 5′-O-DMT-2′-deoxynucleoside-3′(2′-chlorophenyl)-phosphates and protected 5′-O-DMT-2′-deoxynucleoside-3′-O-succinates were prepared as described (Gait *et al.*, 1982b) or purchased from Cruachem, UK (SPS grade). *Syn*-2-nitrobenzaldoxime was bought from Cruachem.

Assembly of oligonucleotides

Sets of round paper disks (~50 Whatman 1 Chr disks, Φ 6 mm, 2.8 mg or 15 – 20 Whatman 3MM disks, Φ 6 mm, 6.3 mg, all properly marked with a pencil) were stacked into each of four glass columns with the aid of a pair of tweezers. The columns were flushed with anhydrous pyridine (5 min) using an argon pressure-controlled solvent delivery system (Gait *et al.*, 1982a, 1982b) adjusted to 1 ml/min.

Addition of the first nucleoside Each of the four protected 2-deoxynucleoside succinates (40 mg each, ~50 μmol) was co-evaporated twice with pyridine in 5 ml pear-shaped flasks on a rotaevaporator or in tubes fitted in a Speed Vac concentrator (Savant, USA). Air was let in through a $CaCl_2$ drying tube and the flasks or tubes were sealed with a silicone-rubber stopper (Gallenkamp, UK). Pyridine (300 μl) was added with a syringe to dissolve each of the 2-deoxynucleoside succinates. The succinate solutions, 200 μl (339 μmol) of a freshly prepared solution of MSNT in anhydrous pyridine (500 mg/ml) and 30 μl (72 μmol) of 1-methylimidazole were mixed with syringes in 1 ml oven-dried micro vials. The total of 530 μl of each activated succinate was injected slowly onto the respective column over a 10 min period and then left without flow for a further 50 min. The four activated succinates were injected in parallel on the four columns. After flushing with pyridine (5 min) the flow was stopped and the capping reagent (0.5 ml), acetic anhydride-pyridine-DMAP, 2:8:1 (v/v/w), was injected to each column and left there for 10 min. The reagent was cleaned out by washing with pyridine (5 min). After further washing with DCE (2 min), the columns were taken apart and the paper disks pressed out with the plunger into a beaker. They were washed by swirling with ether and dried in the air. The disks can be stored at this stage at −20°C in closed vials. One disk from each column was routinely subjected to trityl analysis (Gait *et al.*, 1982a, 1982b).

The elongation cycle. The elongation cycle is similar to the addition of the first nucleoside except for the omission of the capping step (which in our hands has not shown any effect), the use of protected deoxynucleotide-TEA salts instead of deoxynucleoside succinates and shorter reaction times. The quantities of the four protected mononucleotide-TEA salts necessary for all the coupling reactions were dried and redissolved as described for the succinates and aliquots taken for each step. Using 30–50 Whatman 1 Chr disks (or 15–20 3MM disks) per column (~2 μmol of bound nucleoside in total) we injected a mixture of 40 mg (~45 μmol) protected nucleotide-TEA salt in 300 μl pyridine, 200 μl MSNT (500 mg/ml in pyridine) and 30 μl of 1-methylimidazole. For a total of up to 70 Whatman 1 Chr disks (15–20 per column) we used half the volumes per injection. Schematically the elongation cycle is as follows. (i) Sort the disks and place them in the four columns (marked A, C, G and T) (10–30 min). (ii) Flush with DCE (1 min). (iii) Flush with 3% DCA/DCE (90 s). (iv) Flush with DCE (1 min). (v) Flush with pyridine (4 min). (vi) Stop the flow. Inject over 5 min the four activated nucleotides to the proper columns. Leave for a further 10 min. (vii) Flush with pyridine (3 min). (viii) Flush with DCE (1 min). (ix) Stop the flow and press the disks into a beaker containing ether. Decant the ether and dry the disks in the air.

With a total of 100 disks, the cycle time is ~1 h.

Deprotection

After the final elongation cycle, each disk was placed in the bottom of an 0.5 ml Eppendorf tube with the aid of a pair of tweezers. 50 μl of deprotection solution [*syn*-2-nitrobenzaldoxime (0.56 g), dioxane-water (1:1, v/v, 8 ml) and 1,1,3,3-tetramethylguanidine (0.40 ml)] was added and the tubes were mixed with a vortex, centrifuged and left over-night (16 h) at room temperature. The tubes were then placed in a Speed Vac concentrator and the solutions evaporated to near dryness (the disks were kept in the tubes). Concentrated ammonia (100 μl) was added and the tubes were vortexed and placed at 50°C for 5 h. After cooling, each sample was divided into two aliquots which were purified either by h.p.l.c. or polyacrylamide gel electrophoresis. All tubes were placed in a Speed Vac concentrator, evaporated for 30 min with a water pump and then to near dryness with an oil pump. When aliquots were to be purified by h.p.l.c., 0.1 M triethylammonium acetate (TEAA) pH 7.0 (100 μl) was added and the solutions washed by extraction with ether (3 x 300 μl). Such samples can be kept for months at −20°C and injected directly onto an h.p.l.c. column (see below). Whereas the h.p.l.c. purification was done at the level of the 5′-O-DMT-protected oligonucleotide (see below) and followed by detritylation, the purifications by preparative gel electrophoresis were carried out after detritylation.

Detritylation. For detritylation, the vacuum-dried aliquots were dissolved in 80% (v/v) acetic acid (50 μl) and kept at room temperature for 20 min. Water (25 μl) was added and the solution extracted with ether (3 times, 200 μl each) and evaporated in a Speed Vac concentrator.

H.p.l.c. purification

The 5′-O-DMT-protected oligonucleotide mixture in TEAA buffer (40 μl) was injected onto an analytical C-18 reverse phase column (4 x 125 mm; 5 μm, spherical particles). The elution was isocratic for 5 min with 15% acetonitrile in 0.1 M TEAA, pH 7.0 (1 ml/min) and then a linear gradient of 15−30% acetonitrile was applied over 60 min. The pure 5′-O-DMT-oligonucleotide, visible as a major peak, eluted after 32−45 min. The eluted oligonucleotide solution was collected in a 1.5 ml Eppendorf tube and evaporated to dryness in a Speed Vac concentrator. After detritylation (see above) the oligonucleotide was ready for use.

Polyacrylamide gel purification

The detritylated vacuum-dried aliquots (see above) were dissolved in 50 μl of water. Although these oligonucleotide mixtures contain residues of chemicals which disturb polyacrylamide gel electrophoresis, a simple ethanol precipitation was normally sufficient to enable direct application onto a gel. 3 M sodium acetate pH 6.5 (5 μl) and ethanol (150 μl) were added and the mixtures placed at −90°C for 30 min. After centrifugation in a microfuge (15 min, ~13 000 *g*, 4°C) the pellets were washed with ethanol (100 μl) and lyophilised. Alternatively, a rapid microscale ion-exchange chromatography (DEAE-Sephadex A25, Pharmacia Uppsala, Sweden), modified from Wildeman *et al.* (in preparation) was performed for small oligonucleotides (e.g., linkers) for which ethanol precipitation is not efficient. Siliconised Eppendorf tubes (0.5 ml) were punctured at the bottom, tightly plugged with siliconised glass wool and filled with 60 μl of DEAE-Sephadex A25 (prewashed with 3 M TEAA, pH 9 and re-equilibrated with 0.1 M TEAA, pH 9). Most of the liquid was removed from the columns by placing them in 1.5 ml Eppendorf tubes and centrifuging 10 s in a microfuge. The oligonucleotides were bound to the columns by resuspending the resins in the oligonucleotide solutions (50 μl) and incubating 3−5 min at room temperature. Most liquid was removed by a 10 s centrifugation (see above). The resins were subsequently resuspended in 150 μl water and 150 μl 0.1 M TEAA, pH 9 (twice each), followed by a 10 s centrifugation after each washing. Oligonucleotides up to eight nucleotides in length were efficiently eluted with 1 M TEAA, pH 9, whereas longer oligonucleotides (up to 22-mers) required 3 M TEAA, pH 9 for quantitative elution. Routinely, we eluted the oligonucleotides from the columns by resuspending the resins in 150 μl 3 M TEAA, pH 9 and incubating at room temperature for 5 min (twice), followed by a 10 s centrifugation after each elution step. The buffer was removed by evaporation in a Speed Vac concentrator at 45°C. The pellets were dissolved in 100 μl water and dried a second time.

For gel purification the pellets were dissolved in 10 μl of 90% formamide, 0.2% xylene cyanol, 25 mM Tris-borate (pH 8.5), 0.6 mM EDTA and electrophoresed on 20% polyacrylamide (acrylamide-bisacrylamide, 19:1) 8 M urea gels (1 mm thickness). The gels were placed on plates with fluorescence indicator (Merck Silicagel 60F 254, No. 5735) and the bands were visualised by u.v. shadowing, sliced out and extracted with water (three times the volume of the gel pieces) overnight at room temperature. The oligonucleotide-containing supernatants were cleaned from polyacrylamide pieces by centrifugation through siliconised glasswool in Eppendorf tubes punctured at the bottom. Desalting and concentration of the purified oligonucleotides was done either by ethanol precipitation or by binding to DEAE-Sephadex A25 (both as described above except that after DEAE-Sephadex chromatography the evaporation was repeated twice).

5′End phosphorylation of oligonucleotides

For Maxam and Gilbert DNA sequence analysis oligonucleotides were 5′ end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase using standard methods (Maniatis *et al.*, 1982). Linkers and adaptors for cloning, and overlapping oligonucleotides for reconstitution of DNA segments, were phosphorylated according to the following protocol: 0.05−0.5 nmol of oligonucleotide were incubated with 1.0 nmol ATP, 3 pmol of [γ-^{32}P]ATP (5000 Ci/mmol) and 5 units of T4 polynucleotide kinase in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine in 20 μl final volume for 30 min at 37°C.

DNA sequence analysis of oligonucleotides

The chemical cleavage reactions for DNA sequencing were performed according to Maxam and Gilbert (1980) with the following modifications (Jay *et al.*, 1982); G cleavage, 10 min at 37°C with dimethylsulphate; A/G cleavage, 80 min at 45°C with pyridine-formic acid; T/C cleavage, 20 min at 45°C with hydrazine; C cleavage, 20 min at 45°C with hydrazine in NaCl. After ethanol precipitation of the hydrazine reactions, they were treated with 20 μl of acetylacetone for 5 min in 0.3 M sodium acetate at room temperature to eliminate remaining hydrazine before the second precipitation. Analytical polyacrylamide gel electrophoresis was performed using standard conditions. The gels (0.3 mm thickness) were treated 10 min with methanol-acetic acid-water (1:1:8, v/v/v) prior to drying on Whatman 3 MM paper on a slab gel dryer (BioRad) and autoradiographed at room temperature without intensifying screen.

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