Solid-phase methods for sequencing of nucleic acids I. Simultaneous sequencing of different oligodeoxyribonucleotides using a new, mechanically stable anion-exchange paper

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ABSTRACT

A solid-phase method for simultaneous sequencing of large numbers of oligodeoxyribonucleotides has been developed using a new, mechanically stable anion-exchange paper. The excellent mechanical properties of the polymer allow the processing of several paper segments in one reaction vessel or to carry out all necessary operations on a larger area of the paper. In addition, DNA material can be chemically eluted from the new carrier during the piperidine reaction, thus avoiding salt elution of DNA and subsequent ethanol precipitation steps — a prerequisite for sequencing oligonucleotides. The approach involves 7 operations including: i) immobilization; ii) washing; iii) modification; iv) washing; v) sorting of the papers; vi) piperidine reaction and chemical elution and vii) lyophilization. All steps can be carried out in 4 to 5 hours independently of the number of oligonucleotides to be sequenced. It is also possible to sequence small oligonucleotides with 3 to 4 base pairs. The method can be fully automated.

INTRODUCTION

The recent advances of chemical oligonucleotide synthesis — the "filter method" of Frank and Blöcker allows the synthesis of up to 200 different short DNA pieces per week — demand adequately rapid sequencing techniques for oligonucleotides. Fast atom bombardment (FAB) mass spectrometry is sufficient for this purpose, it is, however, only available in a few laboratories and requires a large substrate input (0.5 OD). The fingerprint method or a modified Maxam-Gilbert procedure in solution are too slow.

Classical Maxam-Gilbert sequencing can be carried out much more rapidly and economically on a solid-phase. Chuvpilo and Kravchenko recently reported sequencing of long DNA fragments on Whatman DE 81 paper. A disadvantage of their method is the low mechanical stability of the DE 81 carrier which impedes the manipulation of the paper. Besides that, the method involves an ethanol precipitation step to remove NaCl from eluent, during which short oligonucleotide fragments are lost.

We have developed a rapid, simple solid-phase method for simultaneous sequencing of several oligonucleotides based on a new carrier medium with

anion-exchange properties. This new cellulose support exhibits excellent stability during all operations. Moreover, it fulfills the important requirement of allowing chemical elution of bound DNA. The procedure has the following advantages:
- elimination of all time-consuming precipitation steps
- sequencing of short (down to 4 bp) and longer DNA fragments is possible
- simultaneous sequencing of large numbers (up to 50) of oligonucleotides within a few hours
- suitability for automation.

MATERIALS AND METHODS

[$\gamma^{32}$P]ATP and polynucleotide kinase are commercial products of the ZFK Rossendorf of the Academy of Sciences (GDR) and of Boehringer (FRG). DMS was obtained from Koch-Light (England), KMnO$_4$ from Merck (FRG), formic acid from UCB (Belgium). The oligonucleotides d(TCTA) and d(TCTACA) were synthesized by the diester method on solid-phase, d(GTCAUUCAC) and d(CCCAGTCAGGACGTT) by the phosphate triester method on solid-phase in 5'-3' direction, d(CCU-GGAATT), d(CCB$_5$UGGAATT), d(m$_5$CTGGAAATT) and d(TTCTCTACACAC) by the phosphate triester approach in solution and d(TCGTGGACTCACA), d(TCTCTGGATCCT), d(ITATAGACTGCTT) and d(GCTCGGGGCTGTCTT) by the phosphite-chloridite procedure on solid-phase.

Synthesis of mechanically stable sequencing paper with anion-exchange properties (CCS paper)

10 cm$^2$ Whatman 540 paper was activated with cyanuric chloride according to Hunger and Coutelle and then reacted with 0.5g of 2-bromo-ethylamine hydrobromide in the presence of a mixture of triethylamine and acetonitrile (1:2 v/v). Following 12 h reaction at room temperature, the paper is washed thoroughly with water, blotted between layers of filter paper and dried. CCS paper can be stored indefinitely at room temperature.

Figure 1. Synthesis of the CCS anion-exchange paper for simultaneous sequencing of oligonucleotides

[Diagram of the synthesis process]
Labelling and purification of oligonucleotides

The oligonucleotides were labeled with $[\gamma-3^2P]$ATP and polynucleotide kinase at their 5'-terminus. They were purified by gel electrophoresis on 20% polyacrylamide with or without urea at 50-60°C. Water elution of DNA fragments at 60°C was performed once or twice for 15 min. The aqueous oligonucleotide solution which should contain $3-5\times10^6$ cpm/100μl is ready for application onto the polymeric carrier.

Preparation of CCS sequencing paper and immobilization of oligonucleotides

According to the number ($n$) of oligonucleotides to be sequenced, $4 \times n$ 2-4 mm$^2$ pieces of the paper are cut out and marked with a pencil (Fig. 2, left) or 4 longer paper strips or squares are prepared on which $n$ oligonucleotides can be immobilized at safety intervals of 2-5 mm (Fig. 2, right). 6 oligonucleotides can be placed on a strip of 0.3 x 4 cm and ca. 70 fit on a 5 x 5 cm square. Immobilization is achieved by dropping 0.5-1.0μl labeled oligonucleotide solution onto the carrier following by air drying. This cycle is repeated until 20,000, 80,000, 30,000 or 60,000 cpm, depending on the G, A+G, T>C or C reaction, are bound to the carrier. The papers are then washed twice with water and ethanol to remove salt and urea. All carriers can be washed simultaneously. After each wash the papers are blotted between filter sheets. When large numbers of DNA fragments are to be immobilized, it is convenient to use a multichannel pipetting device.

Simultaneous chemical modification of oligonucleotides in reaction chambers

The paper pieces, strips or squares are placed in 4 reaction chambers (Eppendorf® or larger vessels depending on the size of the carrier) for the 4 modification reactions (G, A+G, T>C, C). The following reagents are added:

G$^6$ : - 200μl sodium cacodylate buffer pH 8 or ammonium formate buffer pH 3.5, 1-2μl dimethyl sulphate
A+G$^5$ : - 80μl 66-88% formic acid

<table>
<thead>
<tr>
<th></th>
<th>G</th>
<th>A+G</th>
<th>T&gt;C</th>
<th>C</th>
</tr>
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<tbody>
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<td>1</td>
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<tr>
<td>5</td>
<td></td>
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</tr>
</tbody>
</table>

G : [ ]
A+G : [ ]
T>C : [ ]
C : [ ]

Figure 2. Preparation of anion-exchange paper for immobilization of 5 oligonucleotides
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T>C\(^{18}\) : - 80\(\mu\)l \(1.3\cdot10^{-4}\) M potassium permanganate freshly diluted from a \(1.3\cdot10^{-2}\) M stock solution

C\(^{18}\) : - 15\(\mu\)l \(H_2O\), 40\(\mu\)l 14 M hydroxylamine pH6, prepared by addition of triethylamine to a hydrochloride solution.

The carrier must be covered by the reaction medium, i.e. the volumes should be increased when necessary. The modification reactions are terminated by removing the papers from the reaction chambers with forceps and washing them twice with water and ethanol. The papers are then dried by pressing them between filter paper sheets and finally air dried.

Simultaneous chemical modification of oligonucleotides on a foil

The reaction solution is dropped on the paper (25-50\(\mu\)l for 10 \(mm^2\) carrier) which should always be completely covered by the solution. Incubation and washing are performed as above.

Piperidine reaction and elution

The reaction with piperidine is carried out separately for each oligonucleotide, i.e. pieces of paper which were treated together during the modification reaction are separated and longer strips or squares are cut apart. The carrier segments are placed in Eppendorf\(^{\oplus}\) tubes and 50\(\mu\)l of 10\% aqueous piperidine are added. After a 30 min incubation at 90° C the papers are removed with forceps and the solutions lyophilized. Lyophilization is repeated twice with 25\(\mu\)l \(H_2O\). The residues are dissolved in 3-5\(\mu\)l formamide containing bromophenol blue and xylene cyanol FF. Gel electrophoresis (0.05 x 20 x 40 cm) on 20\% polyacrylamide with 7 M urea and autoradiography with X-ray intensifying screen over night generates the sequence pattern.

RESULTS

Our procedure is based on a new, mechanically stable two dimensional anion-exchange carrier. The anion-exchange function is determined by a quaternary ammonium group which is covalently bound via a triazine anchor group to mechanically stable Whatman 540 paper (fig. 1, II). The structures of CCS paper II and of the intermediate I (fig. 1) were confirmed by high resolving mass spectrometry and ESCA (data not shown). The carrier has a binding capacity of 120\(\mu\)g/cm\(^2\) for nucleic acids. DNA material bound to CCS paper can not only be eluted with salt solutions (\(NH_4\)HCO\(_3\), \(NH_4\)OOCH\(_3\)C and \(KH_2PO_4\)) but also with 1-10\% aqueous piperidine. The elution efficiency for oligonucleotides (4-17 bp) is 70-90\%. The mechanism of piperidine elution is still uncertain. It seems propable that piperidine induces a Hoffmann elimination at the linkers thus liberating the DNA.
Table 1. Operation cycle of solid-phase sequencing of oligonucleotides on CCS paper

<table>
<thead>
<tr>
<th>No.</th>
<th>Operation</th>
<th>Time (min)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>immobilization of terminally labelled oligo-</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>nucleotides on the carrier</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>wash</td>
<td>2-5</td>
</tr>
<tr>
<td>3</td>
<td>modification reactions</td>
<td>10-20</td>
</tr>
<tr>
<td>4</td>
<td>wash</td>
<td>2-5</td>
</tr>
<tr>
<td>5</td>
<td>sorting of the paper pieces</td>
<td>5-10</td>
</tr>
<tr>
<td>6</td>
<td>piperidine reaction and elution</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>lyophilization</td>
<td>120-180</td>
</tr>
</tbody>
</table>

The solid-phase procedure on CCS paper with its 7 operations shown in tab. 1 takes 4-5 hours, independent of the number of oligonucleotides to be processed. Its efficiency increases the more oligonucleotides are sequenced simultaneously. For modifying the oligonucleotides, the following reactions were selected for the routine: G with dimethyl sulphate, A+G with formic acid, T>C with potassium permanganate, and C with hydroxylamine. The losses during the reactions with excess of modification reactant are 10, 80, 1-10 and 50-80%, respectively. The DNA material released from the paper during the modification reaction can not be bound again and, therefore, does not interfere with the simultaneous sequencing of several oligonucleotides. The expected losses are balanced by immobilizing 4- or 3-fold the amount of oligonucleotide onto the A+G- and the C-paper.

With the help of the solid-phase method developed by us we have sequenced oligonucleotides with chain lengths of 4-16 bp separately as well as simultaneously. The sequence patterns are independent of the chain length. The mode of processing (in reaction vessels or on a foil) and the size of the carrier also have no influence on the quality of the sequence pattern. These facts are illustrated by fig. 3 showing the simultaneous sequencing of a tetra-, a hexa-, and a decanucleotide on a foil, by fig. 4 showing the simultaneous sequencing of three base-modified nonanucleotides on small paper pieces in reaction vessels and by fig. 5 showing the simultaneous sequencing of five pentadecanucleotides on paper strips in reaction vessels.

All oligonucleotides (except the tetramer in fig. 3, left) were also
Figure 3. Simultaneous solid-phase sequencing of three short oligonucleotides: a tetramer (left), a hexamer (middle) and a decamer (right). Lane 1: G (DMS), lane 2: A+G (HCOOH), lane 3: T>C (KMnO₄), lane 4: C (NH₂-OH) and lane 5: T>C (OsO₄). Sequenced by classical Maxam-Gilbert technique in solution and the patterns obtained by the two methods were compared. Significant differences appear when the chain length is less than 10 bp. Sequence patterns of shorter oligonucleo-

Figure 4. Simultaneous solid-phase sequencing of three base-modified nonanucleotides containing uracil (left), 5-bromouracil (middle) and 5-methylcytosine (right). Lane 1: G (DMS), lane 2: A+G (HCOOH), lane 3: T>C (KMnO₄) and lane 4: C (NH₂-OH).
Figure 5. Simultaneous solid-phase sequencing of five pentadecanucleotides. Lane 1: G (DMS), lane 2: A+G (HCOOH), lane 3: T>C (KMnO₄) and lane 4: C (NH₂-OH).

Oligonucleotides sequenced in solution are sometimes only interpretable on the basis of the known primary structure, since the drastic reaction conditions lead to a loss of specificity. Besides that, there are significant losses of input DNA material during the precipitation steps. When patterns of larger oligonucleotides obtained by both methods are compared (fig. 6) there are usually only differences caused by incomplete removal of modifying reagents during precipitation steps (fig. 6, right: lane 4). Such smeared bands sometimes appear during the Maxam-Gilbert routine. The solid-phase method guarantees the quantitative removal of modifying reagents by simple washes. Therefore, its patterns are always "clean", i.e. misleading signals can only be caused by insufficient specificity of a modification reaction.

Our solid-phase sequencing method also allows the detection of the minor nucleosides 5-methyldeoxycytidine, 5-bromodeoxyuridine and deoxyuridine in synthetic oligonucleotides. In contrast to cytosine, 5-methylcytosine does not react with hydroxyl amine (fig. 4, right: lane 4). Both bases, however, show a weak band in the T>C reaction with potassium permanganate (fig. 4, left, middle and right: lane 3). 5-Bromouracil, uracil and thymine cannot be distinguished by their reaction with potassium permanganate. 5-Bromouracil and uracil, however, can be discriminated from thymine by an additional band in the hydroxyl amine (C-)reaction (fig. 4, left, middle and right: lanes 4).
Figure 6. Sequencing of a pentadecanucleotide using the solid-phase procedure (left) and the classical Maxam-Gilbert technique in solution (right). Left: lane 1: G (DMS), lane 2: A+C (HCOOH), lane 3: T>C (KMnO₄) and lane 4: C (NH₂-OH). Right: lane 1: G (DMS), lane 2: A+G (HCOOH), lane 3: A>C (NaOH)⁶, lane 4: T+C (NH₂NH₂)⁶ and lane 5: C (NH₂-OH).

A further useful discriminatory reaction for bromodeoxyuridine and deoxyuridine with NaOH (A>C) leads to liberation of the oligonucleotide material from the carrier and thus, can only be performed in solution.¹⁴

DISCUSSION

Since the properties of the polymer are decisive for the technology of solid-phase sequencing we tested several available two dimensional carrier media. Carrier like DBM- (Transa-Bind™, Schleicher & Schüll) or CCA- (cyanuric chloride activated (fig. 1, 1), ZIM, Academy of Science of GDR) paper which bind nucleic acids covalently at the heterocyclic bases are not suitable for solid-phase sequence analysis. The covalent connection between carrier and base is, in effect, a chemical modification which leads to additional DNA strand breaks and creates unreadable sequence patterns (data not shown). Carriers with anion-exchange properties are more suitable because binding is only by ionic forces between the negatively charged DNA phosphate groups and the positive charges of the carrier. Among the anion-exchange carriers tested by us, DE 81 (Whatman), NA-45 (Schleicher & Schüll), Zetabind™ (AMF Cuno) and
GeneScreenPlus™ (New England Nuclear) only the first two can be used for sequencing, since material immobilized on Zetabind™ and GeneScreenPlus™ can neither be removed by the high ionic strength of various salts nor by piperidine. Due to its excellent mechanical stability, NA-45, consisting of a plastic (polyamide) membrane base in which DEAE groups are embedded, is superior to DE 81 paper but not to our CCS carrier. Even when working very carefully, tearing or rubbing off of active surface layers of the DE 81 paper can not be excluded. NA-45 and DE 81 share the property that DNA can be eluted with NaCl. But this necessitates a subsequent ethanol precipitation after DNA strand break involving a loss of short fragments. Both polymers are, therefore, more suitable for sequencing long DNA fragments.

The CCS paper introduced by us has an excellent mechanical stability which is determined by its Whatman 540 paper base. The fact that the bound DNA material can be eluted easily with piperidine is particularly advantageous since salt elution and ethanol precipitation can be completely avoided.

In order to select modification reactions suitable for carrier-bound sequencing of oligonucleotides on CCS paper from the multitude of available modification reactions we checked the losses during the reactions listed in tab. 2, performed with an excess of reagent. Comparative values for DE 81 paper are given. Because of the high losses all Maxam-Gilbert standard reactions with hydrazine (T+C, C) and sodium hydroxide (A+C) were inapplicable for both carriers. However, Chuvpilo and Kravchenko reported no losses using hydrazine in combination with saturated NaCl and 0.3 M sodium hydroxide during modification on DE 81. One reason for the discrepancy may the amount of reagent used. We find that with a minimum volume of reagent (only to moisten the paper) the yield of bound DNA can be improved. However, this procedure often leads to inadequately modified oligonucleotides and, therefore, irreproducible results. Therefore, we think it necessary to use modification reagents in larger excess. Such a condition is also a prerequisite for automation of the process.

The advantage of the modification reactions chosen by us (G -DMS, A+G - formic acid, T>C - permanganate and C - hydroxylamine) is their speed (10 min for G and 20 for the others) and the fact that they proceed at room temperature. If required, the A+G reaction with formic acid which entails an 80% loss of immobilized DNA can be replaced by the less disruptive reaction with diethylpyrocarbonate at 90° C. From the point of view of automation it appears more simple, though, to balance losses in modification reactions by a larger DNA input on the carrier.
Table 2. Losses of immobilized oligonucleotide* during the modification reaction on two different anion-exchange carriers CCS and Whatman DE 81 paper under an excess of reagent

<table>
<thead>
<tr>
<th>No</th>
<th>Modification reaction</th>
<th>% losses</th>
<th>Suitability for solid-phase sequencing</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CCS</td>
<td>DE 81</td>
</tr>
<tr>
<td>1</td>
<td>G&lt;sup&gt;6&lt;/sup&gt;</td>
<td>5-10</td>
<td>5-10</td>
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<tr>
<td>2</td>
<td>G&lt;sup&gt;21&lt;/sup&gt;</td>
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<td>1-2</td>
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<td>3</td>
<td>A+G&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>95</td>
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<td>4</td>
<td>A+G&lt;sup&gt;21&lt;/sup&gt;</td>
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<td>2</td>
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<tr>
<td>5</td>
<td>A+G&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1(70)</td>
<td>1(90)</td>
</tr>
<tr>
<td>6</td>
<td>A&gt;G&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1,2 M NaOH, 40 min, 60° C</td>
<td>85</td>
</tr>
<tr>
<td>7</td>
<td>T+C&lt;sup&gt;6&lt;/sup&gt;</td>
<td>99</td>
<td>89</td>
</tr>
<tr>
<td>8</td>
<td>T+C&lt;sup&gt;18&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>T&gt;C&lt;sup&gt;18&lt;/sup&gt;</td>
<td>1.3·10&lt;sup&gt;-4&lt;/sup&gt; M KMnO&lt;sub&gt;4&lt;/sub&gt;, 20 min, 20° C</td>
<td>1-10</td>
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<tr>
<td>10</td>
<td>T&gt;C&lt;sup&gt;19&lt;/sup&gt;</td>
<td>0.3% (0.1-5%), 15 min, 0° C</td>
<td>1-10</td>
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<tr>
<td>11</td>
<td>T&lt;sup&gt;23&lt;/sup&gt;</td>
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<td>C&lt;sup&gt;23&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
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</table>

A pentadecanucleotid was used as the reference compound. The losses increase with decreasing chain length. *No specific cleavage at the appropriate base was observed. (+) Not suitable for selfcomplementary oligonucleotides.

Surprisingly, osmium tetroxide does not always generate signals corresponding to the sequence, especially in the case of selfcomplementary oligonucleotides (Fig. 3, right: lane 5) so that the permanganate reaction is preferable due to its greater selectivity. The A+G reaction with piperidine formate<sup>6</sup>, the T+C reaction with hydrazine acetate<sup>18</sup> and both reactions with hydrogen peroxide (C, T)<sup>23</sup> are not applicable for solid-phase sequencing of
short DNA fragments under our conditions due to their lack of specificity.

In summary, we would like to emphasize that the solid-phase technique introduced here allows the simultaneous sequencing of great numbers of oligonucleotides and thus significantly accelerates the sequencing process. The solid-phase sequencing approach is suitable for automation. We are presently developing methods for solid-phase sequencing of long DNA and RNA fragments.

ACKNOWLEDGEMENT

A limited number of the CCS sequencing paper for testing is available at the Academy of Sciences of GDR, Central Institute of Molecular Biology, 1115 Berlin-Buch. The CCS paper can be purchased from Denta-Med-Service GmbH, 1000 Berlin(West)41 (Steglitz), Ermanstrasse 19 (phone 792 05 70). The CCA paper can be purchased from Sartorius GmbH, Göttingen (FRG).

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REFERENCES

20. We often observe misleading bands in the T>C-, G- and C-lane which are situated between the first (\(^{32}\)P phosphate) and the second (\(^{32}\)PNP) signal. The structure of these artefacts is not known yet. These misleading bands occur during both solution and solid-phase sequencing and are, therefore, caused by unspecified modifications. These signals do not impede the reading of the patterns.