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Preparation of cDNA–silica using reverse transcriptase and its DNA sequence determination

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Abstract

A new method for producing macroporous silica (suitable for high-performance liquid chromatography) with covalently attached DNA is presented. The method uses $(dT)_{18}$–silica as a primer, annealed to a poly(A)-RNA template, which is then transcribed using reverse transcriptase. The RNA template is eluted and single-stranded cDNA–silica is recovered. The cDNA–silica can be sequenced using the dideoxy method. These methods provide a facile method for producing cDNA–silica of demonstrable authenticity and provide a unique approach to DNA and RNA sequencing.

1. Introduction

DNA attached to chromatographic supports can be used to provide sequence-specific separation of polynucleotides [1,2]. Such hybrid selection is necessary for many molecular-biology techniques such as subtractive library preparation. DNA–supports are also used for the purification of DNA-binding proteins such as transcription factors [3] (see also Refs. [4,5] for review). Most DNA–supports have been prepared by either adsorption of the DNA onto the support (e.g., DNA dried onto cellulose [6]) or by covalent attachment [4,5]. Both of these approaches have limitations; DNA slowly leaches from the former support, while the latter supports contain DNA which is chemically modified on nucleotide bases by the attachment chemistry used. Once prepared, the DNA–supports are not usually characterized very thoroughly and the DNA is not sequenced.

Recently another approach, the synthesis of DNA–silica [7] and DNA–Sepharose [3] using DNA polymerase, has been reported. In these methods, $(dT)_{18}$ containing a 5′-aminoethyl moiety is coupled covalently to a support using chemistry which reacts through the amino group and does not modify oligothymidylic acid. The $(dT)_{18}$-support is then annealed to a single-stranded DNA–template which contains a 3′-poly(dA) tail and the template-directed sequence is copied directly and covalently onto the 3′-end of the $(dT)_{18}$–support using DNA polymerase [3,7]. For some applications (e.g., subtractive library preparation) an mRNA template would represent a more direct approach. Here we show that RNA can readily be used as a single-stranded template. To characterize the cDNA–silica product, solid-phase sequencing techniques were developed which yield directly the sequence of the copied DNA, thus confirming the identity of the product.

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Reverse transcriptase methods for the production of cDNA–latex [8–10] or cDNA–paramagnetic beads [11] have also recently been described. These materials have proven useful for subtractive library preparation and for preparing probes for identifying tissue-specific genes [8–11]. These studies suggest that cDNA–silica may have these other potential uses and, conversely, that the solid-phase DNA sequencing demonstrated here may also be applicable to these other materials. Here, silica is tested.

2. Experimental

Common molecular-cloning procedures are those described by Sambrook et al. [12] unless stated otherwise.

2.1. Synthesis of RNA

pGEM3Zf(−) containing a 340 bp partial ovalbumin cDNA was restricted by digesting 100 μg with 200 units of *Hind*III for 2 h at 37°C. The resulting DNA was extracted with phenol–$CHCl_3$, ethanol precipitated, and dissolved in 100 μl TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 7.5). RNA was synthesized using a modification of the protocol provided by Promega in a mixture containing 20 μg restricted plasmid, 40 m*M* Tris–HCl, 6 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 2 m*M* spermidine, 10 m*M* NaCl, 1 m*M* of each rNTP, 100 units RNasin, 10 units inorganic pyrophosphatase, and 400 units of T7 RNA polymerase in a volume of 0.2 ml. The mixture was incubated at 37°C for 2 h and then 20 units of RNase-free DNase was added and incubation at 37°C was continued an additional 15 min. The RNA was then extracted with phenol–$CHCl_3$, ethanol precipitated from ammonium acetate, and reconstituted in 50 μl of diethylpyrocarbonate (DEPC) treated, sterile water. This procedure typically gave 100–250 μg RNA.

2.2. Synthesis of $(dT)_{18}$–silica

Synthesis of $(dT)_{18}$–silica was as previously described for bulk synthesis [7] except that 100 nmol of 5′-aminoethyl–$(dT)_{18}$ per gram silica was used. Several different batches of $(dT)_{18}$–silica prepared by this method bound (i.e., via hybridization) between 2.2 and 16.6 nmol/g silica (10.9 ± 6.1, mean ± standard deviation, $n = 4$) of a 5′-end labeled oligonucleotide of sequence GTGGAATTGTGAGCGGATAAC-AATT–$(dA)_{18}$. This will be referred to as the $(dT)_{18}$–silica maximum binding capacity.

2.3. Synthesis of cDNA–silica

To 100 mg of $(dT)_{18}$–silica was added an amount of RNA equal to its maximum binding capacity. In a typical experiment using $(dT)_{18}$–silica with a 12 nmol/g maximum binding capacity, 120 μg of the 340 nucleotide (nt) partial ovalbumin RNA was used. The silica was first washed by centrifugation in RT buffer (50 m*M* Tris–HCl, pH 8.3, 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol) and the RNA was added in 200 μl. The mixture was heated to 65°C for 10 min, then slowly cooled over 30 min to 20°C to anneal the RNA to the silica. The silica was then washed five times by centrifugation (1 min, 14 000 *g* in a microcentrifuge) with 0.8 ml of RT buffer, then five more times with 0.8 ml portions of RT buffer containing 2 m*M* of each dNTP. The supernatant from the last wash was then removed and 24 μl (4800 units) of Moloney murine leukemia virus reverse transcriptase was added and the mixture incubated at 37°C for 1 h. The silica was then washed five times with 0.8 ml of each of the following: RT buffer at 42°C, TE buffer at 42°C, TE buffer at 90°C, and finally water at 90°C. The bulk of the RNA template elutes in TE buffer at 90°C and the amount of RNA eluted in this fraction was used as an initial measure of the amount of cDNA–silica synthesized. This amount ranged from 61 to 175 pmol/g silica.

The amount of full-length cDNA copied onto the silica was determined by the binding of 5′-end labeled OV-S oligonucleotide (GCTAGAAAGCTGTATTGCC) in experiments such as those shown in Table 1. This oligonucleotide is complementary to the 3′-end of the full-length cDNA–silica synthesized. The results obtained agreed well with the capacity

Table 1
The amount of full-length (340 nucleotide) cDNA–silica is determined by hybridization of a 3′-end specific oligonucleotide probe (OV-S)

Condition	Number	Oligonucleotide bound (pmol/g silica)
Added initially		6 610 ± 220[c]
Wash[a]	1	6 380 ± 200
	2	96 ± 17
	3	5 ± 1
	4	4 ± 3
	5	2 ± 0
Elute[b]	1	98 ± 7
	2	17 ± 4
	3	2 ± 0
	4	2 ± 0

[a] Silica washed with 20°C TES buffer (10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 150 m*M* NaCl).
[b] Silica eluted with 90°C water.
[c] Mean ± standard deviation, duplicate samples.

determined from RNA template elution described above.

2.4. Sequencing

For plasmid or silica sequencing, OV-S was the primer. For comparison, the double-stranded pGEM3Zf(−)–ovalbumin plasmid (20 μg) was sequenced following the protocol suggested in the Sequenase 2.0 manual (US Biochemicals). For silica, an amount of silica containing 1 pmol of cDNA was used. For the experiment shown in Fig. 2, this silica had 175 pmol cDNA/g silica and 5.7 mg of silica was used. The silica is washed five times with 0.2 ml of Sequenase buffer (10 m*M* Tris–HCl, pH 7.5, 20 m*M* $MgCl_2$, 50 m*M* NaCl) by centrifugation for 1 min at 14 000 *g*. The pellet was resuspended to a volume of 20 μl and 1 μl of 165 μ*M* OV-S primer was added, and the mixture was heated to 65°C for 2 min, slowly cooled to 20°C, and placed on ice. Then, 1 μl of 0.1 *M* dithiothreitol, 2 μl of a mixture containing 7.5 μ*M* each of dGTP, dCTP, and dTTP, 2.5 μl (25 μCi) of α-^{35}S-dATP (1308 Ci/mmol), and 2 μl (3.25 units) of Sequenase (T7 DNA polymerase) was added. The mixture was incubated 5 min at 20°C, 2 μl additional Sequenase was added, and 7.5 μl portions were delivered to each of four tubes containing 2.5 μl of the appropriate dideoxy termination mix supplied with the Sequenase. The four reactions were incubated at 37°C for 5 min and then 6.7 μl of Stop solution was added. The samples were heated to 90°C for 2 min immediately before loading 1.5 μl onto the acrylamide gel.

3. Results

The method used to prepare cDNA–silica from a $(dT)_{18}$–silica and template RNA is depicted in Fig. 1. The template RNA was pro-

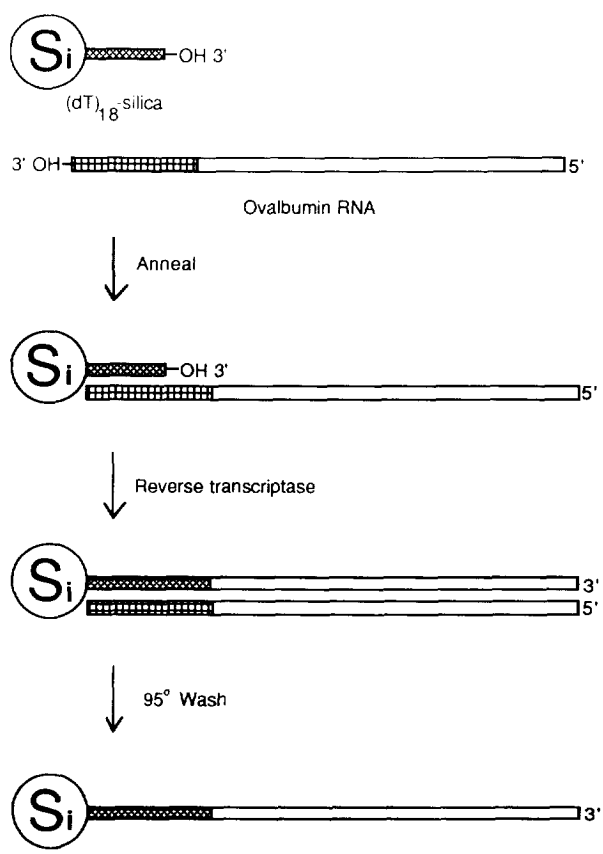

Fig. 1. Synthesis of cDNA–silica is shown diagrammatically. Silica (encircled "Si") containing 5′ coupled $(dT)_{18}$ (crosshatched) hybridizes (anneals) to a poly(A) (different crosshatch) RNA and primes synthesis of a cDNA sequence complementary to the RNA template.

duced from a 340 nt fragment of ovalbumin cDNA cloned into the *Eco*R I site of the pGEM3Zf(−) plasmid just downstream of the plasmid's T7 RNA polymerase promoter. The plasmid was linearized by restriction at a downstream *Hind*III site and transcribed with T7 RNA polymerase. The RNA produced contains 305 nt of ovalbumin coding sequence at the 5′-end and a 3′ poly(A) tail approximately 35 nt in length. After the RNA is produced, the plasmid template is destroyed with DNAase. Purified RNA is then annealed to $(dT)_{18}$–silica, washed thoroughly, and reverse transcriptase is added to copy the template-directed sequence onto the silica. The RNA template is then eluted and washed away at 90°C and the RNA recovered provides an initial estimate of the amount of cDNA–silica synthesized. Using the methods described, silica containing between 61 and 175 pmol cDNA per gram was obtained.

The amount of full-length cDNA on the silica can be determined using a radiolabeled oligonucleotide probe which is complementary to the 3′-end of the synthesized cDNA. In this case, an oligonucleotide called OV-S was used which binds to nucleotides 21–40 from the 3′-end of the full-length cDNA. Results obtained in a typical experiment are shown in Table 1. When excess 5′-end labeled OV-S is added, 98 pmol of the oligonucleotide probe binds (hybridizes) per gram silica and subsequently elutes. To accurately determine the amount of full-length cDNA, the probe which binds at high stringency should be determined; i.e., washes at low salt or high temperatures should be used to remove any non-specifically bound probe. However, the simple protocol used in Fig. 1 works as well as more complex procedures where higher-stringency washes are used prior to elution and gives the same capacity, indicating little non-specific binding under these conditions. The capacity determined by RNA template elution or by binding the OV-S oligonucleotide probe specific for the 3′-end of the cDNA synthesized are not significantly different (data not shown), demonstrating that most of the cDNA–silica is full length.

The cDNA silica can be sequenced as shown in Fig. 2. In this figure we show sequencing using Sequenase. In other experiments (data not shown) we have obtained similar results using *E. coli* DNA polymerase, Klenow large fragment. For comparison, the double-stranded plasmid containing the partial ovalbumin cDNA is also shown. The two sequences shown were taken from different experiments simply because plasmid sequencing with this plasmid/primer combination did not always work in the experiments where the silica yielded sequence. The sequence from silica not only proves its authenticity but

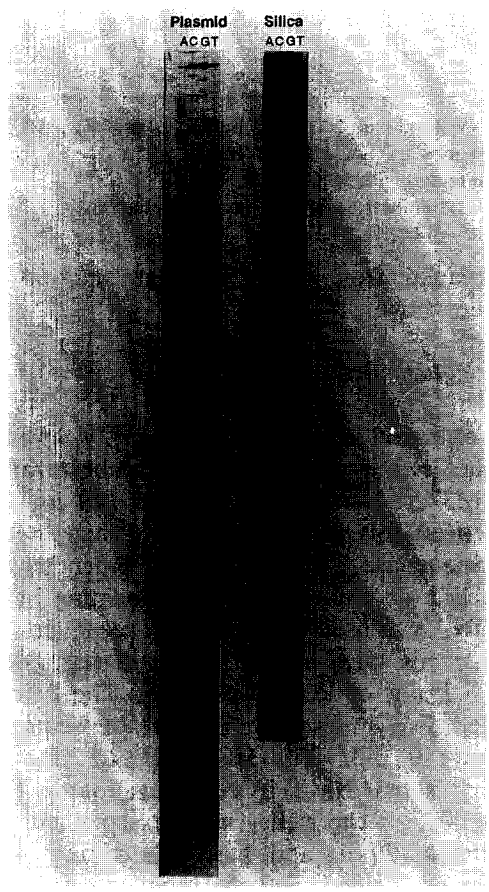

Fig. 2. Comparison of DNA sequencing on plasmid and silica. The autoradiograms obtained in separate experiments for cDNA–silica and the pGEM3Zf(−) plasmid containing the partial ovalbumin sequence are shown. In both cases, OV-S was the oligonucleotide primer and sequencing used the dideoxynucleotide method with Sequenase 2.0 as described in Experimental.

also proves how the cDNA is attached. The longest sequencing fragments (at the top of the gel) show the poly(A) region which was hybridized to the silica and immediately following this the sequence stops, showing that silica attachment, as expected, immediately follows the poly(A) tail region. In contrast, the sequence from the plasmid continues past the poly(A) tail, into (downstream) plasmid sequences. Our experience has been that sequencing from silica has always given results as clear as that obtained from plasmid, and frequently much better.

4. Discussion

We have shown that reverse transcriptase and RNA templates provide a convenient means for the enzymatic production of DNA–silica. Somewhat similar techniques have also been reported for paramagnetic beads [11] and latex [8–10]. These other materials were developed for use in identifying tissue-specific genes by preparing specific probes or subtractive libraries. Such methods rely on high-resolution fractionation of cellular RNA by hybrid selection; however, because of poor mass transfer characteristics of the chosen supports, resolution is limited. The macroporous silica used here is suitable for high-performance liquid chromatography and could potentially improve this fractionation. Our previous studies using oligonucleotide–silica supports have shown that single base differences between oligonucleotides allow separation by temperature, salt, or formamide gradient elution [1,2]. We have also demonstrated that enzymatically prepared DNA–silica is highly selective. DNA–silica specifically binds complementary sequences and shows little binding of non-specific DNA sequences [7]. Furthermore, enzymatically prepared DNA–Sepharose [3] and DNA–silica (unpublished data) has been shown to be an extremely selective chromatographic medium for transcription factors, allowing rapid purification to a high state of purity. The high resolution indicated by these studies suggest that the cDNA–silica prepared here could markedly improve hybrid selection experiments, a hypothesis we are currently testing.

For preparing cDNA–silica for uses such as subtractive library construction, an isolated, naturally occurring mRNA would provide the most logical template choice. For other uses where a DNA is a more practical template, other methods based upon DNA polymerases have already been described [7].

The amount of DNA copied onto silica is clearly adequate for many uses. For example, we routinely use small (30×4.6 mm I.D.) columns which contain 0.3 g silica. Such a column would contain about 30 pmol cDNA which is in the proper sense for purifying about 19 μg of full-length (i.e., 1.9 kb) ovalbumin mRNA. This is an amount of mRNA sufficient for northern blots, library construction, etc.

DNA sequencing of the cDNA–silica support was developed here initially to demonstrate that the full-length sequence was faithfully copied, a necessary characterization of a new support which has been lacking from other reports of DNA–support synthesis. However, this solid-phase sequencing technology has useful attributes not shared by liquid-phase sequencing. For example, after the Sequenase reactions, the silica can be washed and the DNA termination fragments selectively eluted or eluted in smaller volumes. This would allow removal of excess radioisotope, for the buffer components to be changed from those needed for Sequenase to those more appropriate to gel electrophoresis, or could be useful to concentrate the sample prior to electrophoresis. Solid-phase chemistry is also often more easily automated since the DNA is more easily recovered after each step. Protein sequencing is routinely performed on proteins which remain immobile on a solid support because of the advantages inherent in this approach; DNA sequencing may also benefit from solid-phase approaches.

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