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Review

Affinity chromatography with nucleic acid polymers

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ABSTRACT

Column chromatography utilizing polynucleotides immobilized on solid supports is reviewed. This form of affinity chromatography is used for the isolation of polynucleotides and polynucleotide binding proteins, and to a lesser extent for analysis. Several specific applications within these categories have been widely used in the biomedical sciences. Poly(A) mRNA is routinely isolated using oligo(dT) or oligo(dU) supports. Many DNA binding proteins, including transcription factors, restriction endonucleases, and proteins involved in DNA repair, replication, recombination, and transposition have been purified using DNA affinity chromatography. Recently, DNA supports suitable for use in high-performance liquid chromatography have been described and utilized. The current usage of DNA affinity chromatography is reviewed and potential future uses for this technology are speculated upon.

CONTENTS

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LIST OF ABBREVIATIONS

dA	Deoxyadenosine
dC	Deoxycytidine
DEPC	Diethylpyrocarbonate
dG	Deoxyguanosine
dI	Deoxyinosine
dNTP	Deoxynucleoside triphosphate
dsDNA	Double-stranded deoxyribose nucleic acid
dT	Deoxythymidine
$(dT)_{18}$-silica	Octadecamer thymidylic acid coupled to macroporous silica
dU	Deoxyuridine
EDTA	Ethylenediamine-N,N,N′,N′-tetraacetic acid
HPAC	High-performance affinity chroma tography
HPLC	High-performance liquid chromatography
IPTG	Isopropylthiogalactoside
LPAC	Low-pressure affinity chromatography
mRNA	Messenger RNA
NAG	N-Acetylglucosamine
NHS-silica	N-Hydroxysuccinimidyl ester derivative in macroporous silica
PCR	Polymerase chain reaction
rRNA	Ribosomal RNA
ssDNA	Single-stranded DNA

1. INTRODUCTION

Chromatography utilizing immobilized nucleic acids is a broad field; here we will focus only upon column-based, affinity chromatography which uses polymers of nucleic acids (typically DNA) immobilized upon a solid support. The uses of such columns for the purification of polynucleotides and polynucleotide binding proteins will be reviewed and the other potential uses of these materials will be discussed. Furthermore, wherever possible, we will emphasize techniques shown to be of high resolution and ones applicable to high-performance liquid chromatography (HPLC). An exhaustive review has not been attempted but rather current literature exemplifying new chromatographic approaches has been emphasized.

By limiting the review in this way, we are

exluding other chromatographic modes such as ion exchange or reversed phase which are often used for the separation of mononucleotides and polynucleotides. Several reviews found in this volume deal with these topics. By limiting the review to separations involving polynucleotides, we exclude the use of immobilized mononucleotides in protein purification. Several mononucleotides or dinucleotides (*e.g.*, ATP, NAD, etc.) are coenzymes and supports containing these compounds have frequently been used to purify the enzymes which use them. While this is certainly affinity chromatography, this topic has been dealt with at length in the numerous reviews and monographs dealing with affinity chromatography and need not be discussed further here.

2. BACKGROUND

2.1. *Affinity chromatography of polynucleotides and binding proteins, general aspects*

In its broadest sense, affinity chromatography is any chromatography which uses a specific chemical or biological affinity of two substances as the basis for a chromatographic separation. In the case of polynucleotides, there are two kinds of "specific affinity" which may be exploited. The more basic of the two involves the base pairing of the nucleotide bases between two complementary polynucleotide strands; this affinity provides a powerful, sequence-specific separation technique for polynucleotides. The other involves the affinity of polynucleotide binding proteins for DNA or RNA; this affinity allows the separation of these proteins based upon their ability to bind polynucleotide sequences. Both will be the subject of this review.

2.1.1. *Polynucleotides*

The naturally occurring polynucleotides are DNA and RNA. In cells, DNA is usually double-stranded while RNA is usually single-stranded, but both can exist in either double- or single-stranded forms. The base pairs which form between strands are not of equal strength; adenine–thymine (A–T) base pairs are the weaker and involve two hydrogen bonds between the bases while cytosine–guanine (C–G) pairs involve three hydrogen bonds. The nature of ribose also affects stability. RNA–RNA duplexes are more stable (*i.e.*, dissociate or "melt" at a higher temperature), followed by RNA–DNA hybrids, with DNA–DNA duplexes being the least stable. This base pairing provides the basis of one form of polynucleotide affinity chromatography. By constructing columns with a single-stranded polynucleotide attached to the support, single-stranded polynucleotides which can base pair with the attached sequence will be retained by the column. Base pairing (*i.e.*, hybridization, annealing) occurs most effectively between two strands with complementary (*i.e.*, antisense) sequences. For example, a column attached $(dT)_{18}$ will form the most stable hybrid with an $(A)_{18}$ or longer oligomers. Any mismatches in sequence between the two strands leads to a less stable hybrid. Mismatches that occur at the ends of the strand are less detrimental than those occurring intrachain, but both lower hybrid stability. Hybridization is also directional, *i.e.*, two complementary polynucleotide strands only base pair when aligned in an antiparallel fashion. The sequence on the column (*e.g.*, 5′ to 3′) must have a complementary, antiparallel sequence which will allow base pairing with the applied strand (*e.g.*, 3′ to 5′); the "backward" sequence can be synthesized but will not hybridize. Base pairing is diminished by raising temperature (to "melt" the hybrids as a consequence of increased thermal motion), lowering salt concentration (the phosphate anion of the poly(deoxy)ribose phosphate backbone repel the two strands as salt in decreased), or increased concentrations of denaturants such as formamide and these provide strategies for eluting the hybridized strand. Thus, base pairing provides a sequence-specific separation mechanism for polynucleotide chromatography. Many of these concepts are shown diagrammatically in Fig. 1.

2.1.2. *Polynucleotide binding proteins*

Proteins which bind to RNA or to double- or single-stranded DNA are of great research interest for reasons which will become clear below. The

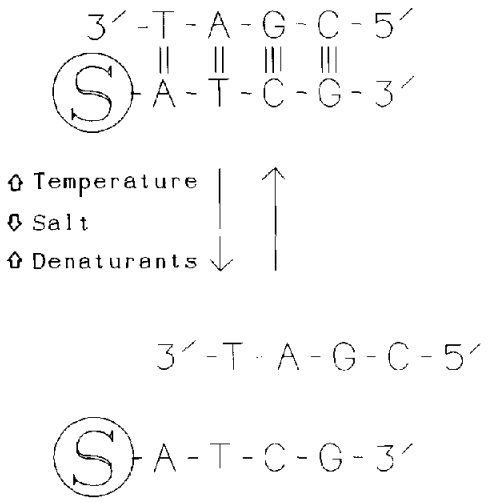

Fig. 1. Hybridization providing the basis for one kind of polynucleotide affinity chromatography. Shown are the four kinds of bases found in DNA arranged on two strands to show how base pairing occurs. The vertical lines indicate the number of hydrogen bonds involved in base pairing. The antiparallel orientation of hybrids as well as the effect of salt, temperature, and denaturants on hybrid formation are also depicted. The large, encircled "S" used in this figure and others denotes a chromatographic support to which the DNA strand is immobilized, here by way of the 5′-end of the DNA.

purification of these proteins using sequence-specific DNA-Sepharose has been reviewed [1]. To separate these proteins, either single- or double-stranded columns may be needed depending upon the protein of interest. Also, some of these proteins bind a specific sequence (*e.g.*, TATA) while others are much less fastidious and must bind many different sequences (*e.g.*, genomic DNA) as a part of their function. Thus, columns containing specific sequences and those containing heterogeneous mixtures of many different sequences are often required; ways for making each of these types of columns have been reported and will be described.

2.2. Low-pressure affinity chromatography (LPAC)

Gilham [2] pioneered the affinity chromatography of polynucleotides. Oligothymidylic acid, oligo(dT), was prepared by polymerizing the monomer (TMP) in the presence of a carbodiimide, cellulose was then added, and the polymer attached by reaction of the 5′-end phosphoryl with the hydroxyls of cellulose. A column prepared from this material was shown to separate various oligomers of adenylic acid in a temperature-dependent fashion [2]. This first DNA-cellulose also has turned out to be one of the most widely used of all the DNA supports synthesized. Oligo(dT) supports hybridize (*i.e.*, base pairs) with the 3′-polyadenylic acid "tails" found on most eukaryotic messenger RNA (mRNA). Oligo(dT)-celluloses, -sepharoses, and -agaroses are widely used for poly(A) mRNA isolation [3,4].

Other homopolymeric DNA supports have since been made including oligodeoxyadenylic (dA), -cytidylic (dC), -guanylic (dG), and -uridylic (dU) acid derivatives of cellulose [5] and agaroses. Oligo(dU) supports can be used as a substitute for oligo(dT) supports in mRNA isolation. The dA, dC, or dG nucleic acids contain potentially reactive amine substituents (*e.g.*, the 6-aminopurine of adenine, etc.) which must be chemically blocked prior to polymerization and support coupling and deblocked afterwards. Suitable blocking/deblocking chemistry has been described for cellulose [5] but this chemistry would not be suitable for some other supports. Deblocking is usually accomplished using concentrated aqueous ammonia solutions which, while applicable to celluloses and agaroses, would be detrimental to high-performance silica supports because of the solubility of silica in alkaline solutions. More will be said of this later in sections dealing with choice of support and coupling chemistries.

Heteropolymeric DNAs containing all four of the common DNA nucleotides (*i.e.*, dA, dT, dC, and dG) of course have the greatest diversity and are the DNAs of greatest general interest. These DNAs can represent restriction endonuclease cleavage sites, the binding sites for other DNA binding proteins, probes useful for isolating a specific DNA or RNA, etc. and have many uses which cannot be filled by the homopolymeric DNAs. These have also been coupled to celluloses [6–18], agaroses [1,8,17,19–40], a variety of other media [41–44], and most recently macroporous

silica for HPLC [45]. The coupling schemes which have been used will be outlined further below, but these can be conveniently divided by what provides the point of attachment of the support to the DNA chain and whether single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) is attached. The original oligo(dT)-cellulose described by Gilham [2,5] and more recently DNA coupling to macroporous silica [45–48] are supports coupled to the 5′-terminus of an ssDNA. Several other ways of making these columns also result in a single attachment at a terminus (either 3′ or 5′) of a single strand as will be described below. Supports of this type have wider uses since they can be made double-stranded by hybridizing the complementary strand, can be used single-stranded, and the nucleotide bases are available for the affinity interaction in an unmodified form. Methods which yield only double-stranded supports or which modify the nucleotide bases have more limited uses but have been widely used for some applications. We will discuss these various types below under coupling chemistry.

Supports can be prepared by coupling either DNA or RNA, but the overwhelming number are DNA supports. There are three important reasons why. First, the chemical synthesis of DNA is much easier since DNA lacks the 2′-hydroxyl of RNA which must be blocked and deblocked for RNA synthesis. The second reason involves the nucleophilicity of the 2′-hydroxyl. Since most coupling procedures currently in use involve reaction with a nucleophile on the DNA or RNA, the presence of the 2′-hydroxyls would allow multiple sites of attachment in some coupling schemes which is often undesirable. The final reason is perhaps most important. Nucleases specific for the digestion of DNA or RNA are common in research laboratories. They can be released from microbes which contaminate mobile phases or from the normal *flora* and *fauna* of human skin. These nucleases are almost impossible to avoid without strenuous precautions. However, all known DNA nucleases (DNases) require a divalent metal ion for catalysis (typically Mg^{2+}, Ca^{2+}, or Zn^{2+}) and are inhibited by chelators such as EDTA. Thus, the simple precaution of adding EDTA to mobile phases and storage buffers is sufficient to prevent the degradation of DNA supports. The RNases are a much more heterogeneous group of enzymes; there is no inhibitor known which is effective against all RNases. Thus, protecting an RNA support from degradation would be a formidable task. Since either DNA or RNA can form base pairs with itself or the other (*i.e.*, DNA–DNA, RNA–RNA, and DNA–RNA duplexes are all stable) and many proteins which bind to ssRNA will also bind ssDNA, there is seldom a case where a DNA support does not provide a more workable alternative to an RNA support.

2.3. High-performance affinity chromatography (HPAC)

Recently, HPLC-based affinity chromatography of polynucleotides has been introduced [45–49]. Two different basic approaches have been taken: in one [49], vinyl or methacrylate derivatives of adenine or thymine bases were attached to a methacrylate derivative of macroporous silica by free radical copolymerization. Notice that in this case, only the nucleic acid bases (without the sugar phosphates) are attached and the polymer coating the silica is not a polynucleotide but rather a synthetic polymer containing nucleic acids. Four different homopolymeric silicas were produced and three of these were able to separate mononucleotides, dinucleotides, and homooligomers up to about twelve in length. Resolution of RNA oligomers differing by a single nucleotide in length or differing in the phosphorylation state of the 2′,3′-terminus was demonstrated [49].

In the other approach, $(dT)_{18}$ containing an amino group on the 5′-end was coupled to macroporous silica using this group to provide a single 5′-point of attachment [46]. While various coupling schemes have been tried, the most satisfactory one overall has been to introduce either a 5′-aminoethylphosphoryl or -aminohexylphosphoryl into a synthetic DNA oligomer and couple this to an N-hydroxysuccinimidyl ester derivative of silica (NHS-silica). Uncoupled NHS-silica subsequently reacts with water [50] to yield a carbox-

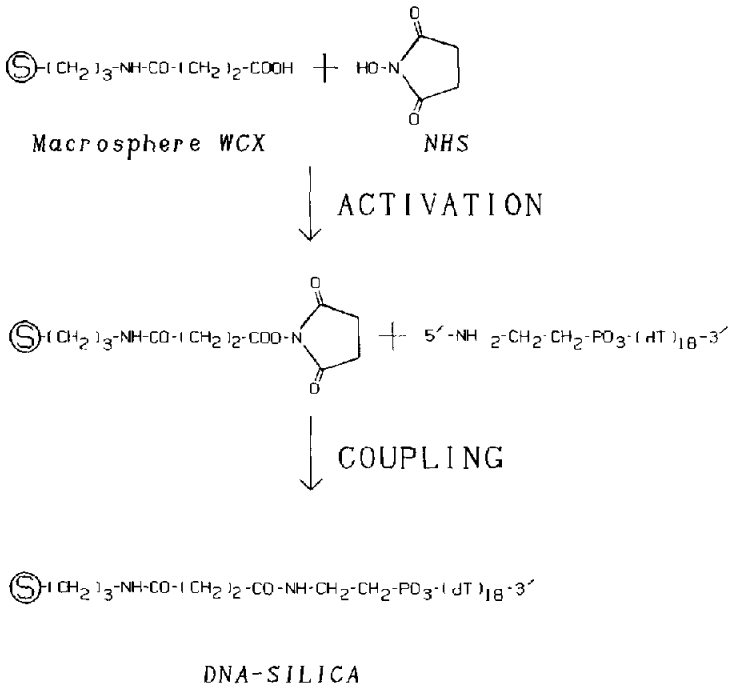

Fig. 2. Coupling of 5′-aminoethylphosphoryl-$(dT)_{18}$ to macroporous silica. The upper part of the figure shows the activation of Macrosphere WCX silica, performed in the presence of a carbodiimide [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide] to give NHS-silica which was then reacted with 5′-aminoethylphosphoryl-$(dT)_{18}$ to yield the final DNA-silica. These reactions were all carried out under flow inside a prepacked HPLC column (30 mm × 4.6 mm I.D.). This figure is taken, with permission, from ref. 46 which should be consulted for further details.

ylate anion which probably repels DNA and prevents it from interacting with the derivatized silica surface. This repulsion insures that the DNA extends out into the mobile phase. The coupling scheme is illustrated in Fig. 2. The support used, Macrosphere WCX, is available commercially (Alltech Assoc., Deerfield, IL, USA) and the activation chemistry has been described [46,50]. Published procedures for coupling DNA inside prepacked columns [46] and for bulk support synthesis [45] are available. Suitable reagents for introducing the 5′-amino moiety during normal phosphoramidite DNA chemical synthesis are available (*i.e.*, the AminoLink reagent from Applied Biosystems, the AminoModifier reagents from Cruachem, Sterling, VA, USA, etc.) and can be incorporated on the last (5′-end) cycle by any competent DNA synthesis facility with little modification of existing protocols.

When 5′-aminoethyl-$(dT)_{18}$-silica was tested, it could separate all oligoadenylates from length of six to eighteen and could resolve oligomers differing by a single nucleotide in length [46]. Fig. 3 shows seven such separations of $(dA)_{12}$–$(dA)_{18}$ oligomers, in this case using temperature gradients; similar resolution has also been demonstrated using salt [46] and formamide [47] gradients.

More recently, methods for the enzymatic synthesis of DNA-silicas have been described [45]. One of these uses a 5′-amino-oligomer as a primer in the polymerase chain reaction (PCR) to produce a dsDNA which is then coupled to silica primarily through the 5′-amino moiety. This strategy is diagrammed in Fig. 4. The other uses $(dT)_{18}$-silica and a 3′-poly(dA) tailed template DNA. The $(dT)_{18}$-silica is hybridized to the template and the Klenow large fragment of *Escherichia coli* DNA polymerase I is used to copy the template directed sequence covalently onto the 3′-hydroxyl terminus of the $(dT)_{18}$ of the silica. The template strand is left associated for applications requiring a dsDNA column or can be

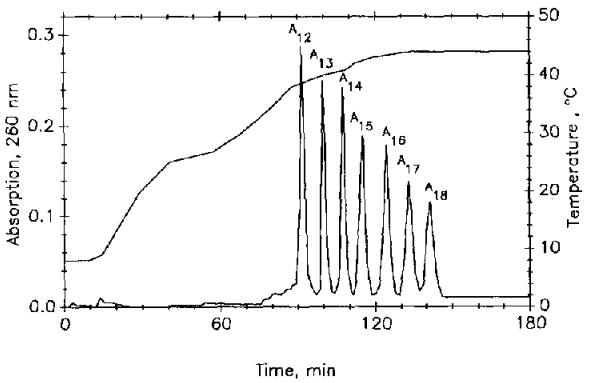

Fig. 3. Resolution of a $(dA)_{12-18}$ mixture using temperature-dependent elution on $(dT)_{18}$-silica. The column, 30 mm × 4.6 mm I.D., 30-nm-pore silica containing 73 nmol (*i.e.*, 11 A_{260} units) of $(dT)_{18}$, was loaded with 1.3 nmol (*i.e.*, 0.3 A_{260} units) of a mixture of oligoadenyates from 12 to 18 in length. The column was loaded at 8°C and eluted by manually adjusting the temperature of a water bath (shown on the right hand ordinate) in which the column was immersed. The mobile phase was 0.49 *M* NaCl, 0.01 *M* sodium phosphate, pH 6.5 at a flow-rate of 0.2 ml/min throughout. Taken, with permission, from ref. 46.

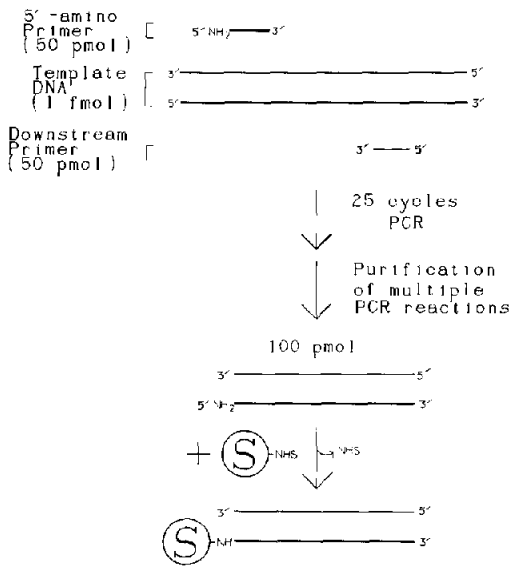

Fig. 4. Coupling of a PCR-produced duplex DNA containing a 5'-amino on one strand to silica. Shown is the scheme used in ref. 45. Multiple PCR reactions were performed using a 5'-amino oligonucleotide "upstream" primer and a downstream limiting primer to copy at 242-mer sequence from a template (*Pvu* II restriction digest of the pGEM-3Z plasmid). The DNA from the PCR reactions was purified and yielded 100 pmol which was then coupled to NHS-silica to prepare a column which was tested successfully. See ref. 45 for further details.

eluted to make the column ssDNA; the eluted strand can be reused as a template for further enzymatic DNA-silica synthesis [45]. These methods allow essentially any DNA, artificially or naturally derived, to be attached or copied directly onto silica. This strategy is depicted in Fig. 5.

3. CURRENT USES

There are two broad uses which can be identified as described above: DNA supports are used to fractionate polynucleotides and to fractionate polynucleotide binding proteins.

3.1. Polynucleotide separations

By far, the most frequent usage of this type is for the isolation and, to a lesser extent, the analysis of poly(A) mRNA. For this purpose, oligo(dT) or oligo(dU) supports have been used [3,4].

Nearly all mRNAs (histone mRNAs are notable exceptions) from eukaryotes contain long poly(A) tracts at their 3'-end. The maximum length of these "tails" varies with species being about 50 nucleotides in *Saccharomyces* [51,52], somewhat longer in *Dictyostelium* [53], and usually around 200 nucleotides in length in mammals [54]. Since mRNA is only a small fraction of cellular RNA (~5%) but is the RNA of most interest to much of molecular biology, these poly(A) tails provide a convenient way to purify mRNA. For this purification, oligo(dT) or oligo(dU) columns containing oligomers of about 12–18 nucleotides in length provide sufficiently stable hybrids with the poly(A) mRNA to allow purification at room temperature and moderate ionic strengths. For mRNA isolation, the salt dependence of hybridization usually forms the basis of the separation. Thus, RNA is loaded onto the column at NaCl concentrations around 0.5 *M*, the column is washed with this buffer, and finally eluted with a buffer of low ionic strength [4]. Frequently, chromatography must be repeated to obtain sufficient purity.

The analysis of poly(A) tail length has also been a less frequent use of these columns. Poly(A) tails are at their maximum length immediately after

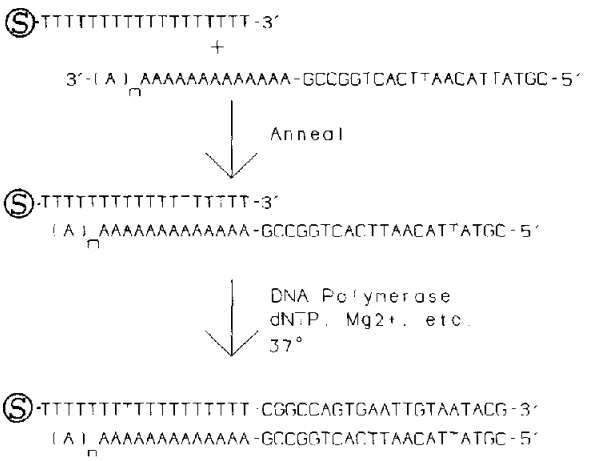

Fig. 5. Template-directed, enzymatic synthesis of a DNA-silica. Shown is the scheme used in ref. 45. A 21-mer sequence was 3′-end poly(dA) tailed using the terminal deoxynucleotidyl transferase reaction and served as the template for template-directed DNA-silica synthesis. The template was annealed to $(dT)_{18}$-silica and the Klenow large fragment of *Escherichia coli* DNA polymerase I was used to copy the complement of the template sequence onto the 3′-end of the covalently attached $(dT)_{18}$. The silica was used to prepare a column which was subsequently shown to contain the expected sequence. See ref. 45 for further details.

synthesis and are shortened during their lifetime in the cell [52,53,55]; when the poly(A) tail is removed, mRNA is degraded more rapidly (for review, see ref. 56). Cells contain poly(A) binding proteins which appear to function in protecting the poly(A) tails from shortening [54,57]. The rate at which poly(A) tails change for a particular mRNA is a function of numerous environmental factors including the hormones, nutrients, and other conditions to which a cell is exposed [55,58,59]. Poly(dU)-Sepharose has been particularly useful in these studies of factors influencing poly(A) tail length. For these purposes, the poly(A) mRNA is applied to the column and the column is eluted by increasing temperature. The longer poly(A) mRNA hybrids elute at higher temperatures and elution temperature thus provides a rough measure of poly(A) tail length [55].

Recently, these methods have been adapted to the HPLC [47]. Using $(dT)_{18}$-silica, mRNA could be purified from *Saccharomyces* RNA in as little as 8 min using a combination of salt- and temperature-dependent elution; such a separation is demonstrated in Fig. 6. The isolated mRNA was of the expected length and was shown to be free of rRNA contamination. $(dT)_{50}$-silica has been used to resolve mRNA based upon poly(A) tail length [47].

3.2. Polynucleotide binding proteins

Another wide use for DNA supports has been in the study of polynucleotide binding proteins. One promising use has been in the purification of restriction endonucleases [27,32], important tools in molecular biology. These enzymes hydrolyze dsDNA at precise sequences within the DNA duplex. The recognition sequence is typically from 4 to 8 base pairs in length. Some of these endonucleases bind their recognition sequence in the presence or absence of Mg^{2+} but are catalyt-

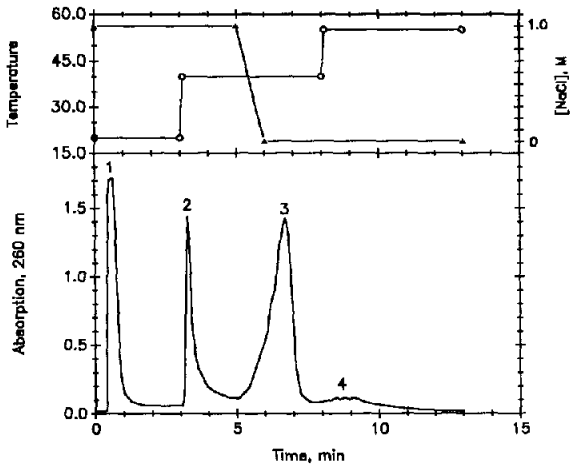

Fig. 6. Rapid isolation of poly(A) mRNA using a combination of temperature- and salt-dependent elution. A 100-μl aliquot containing 6.7 A_{260} units of LiCl-precipitated *Saccharomyces* RNA in 1 *M* NaCl was injected onto a 30 mm × 4.6 mm I.D. $(dT)_{18}$-silica column and rapidly eluted. The salt and temperature gradients used are shown in the upper portion of the figure. The first peak to elute was shown to contain rRNA and non-polyadenylated mRNA while the second peak contains mRNA with only short stretches (< 15) of poly(A). The third peak, containing 2.2 A_{260} units (~88 μg), contained the bulk of the poly(A) mRNA (with 15 < tail lengths < 80). The fourth peak contained mRNA with very long poly(A) tails (length about 80). Taken, with permission, from ref. 47 which should be consulted for further details.

ically active (*i.e.*, digest DNA) only in its presence. This is fortuitous for their chromatography on DNA supports. *Eco* RI [27,32] and *Sph* I [27] have been obtained in highly yield and highly purified using DNA affinity chromatography alone for purification.

However, most of the uses of DNA affinity chromatography have involved other DNA binding proteins. This topic requires some background.

Both RNA and DNA binding proteins can be purified using similar techniques but to simplify this discussion, we will focus only on the DNA binding proteins which have been the subject of much greater study. DNA affinity chromatography has been used to purify proteins which are (1) restriction endonucleases [27,32], (2) transcriptional enhancer/promoter proteins [1,8,12,14–16, 18,19,22–25,28–30,33,35–39,42,44], and proteins involved in (3) transposition [10,40], (4) recombination [9], (5) DNA repair [11], and (6) DNA replication [7,13,16,17,21,31,34,36]. Notice that within these categories, there are ssDNA binding proteins (*e.g.*, categories 4, 5, and some in 6), dsDNA binding proteins (*e.g.*, 1–3 and 6), those which must bind to a specific DNA sequence (1–3) and those which must be able to bind DNA with much less sequence specificity (4–6). These various DNA binding proteins are interrelated in complex ways which can be shown by picking transcription as an example.

The regulation of transcription is an area of intense research and involves various DNA binding proteins including specific and general promoter and enhancer binding proteins (see refs. 60–63 for review). These DNA binding proteins, often referred to as transcription factors, generally bind upstream of the protein coding regions of genes and increase the frequency of transcription to produce its messenger RNA. The study of these various DNA binding proteins has revealed an incredible level of complexity. For example, in the transcription of the actin gene at least ten proteins associate with DNA and the RNA polymerase to make the active, transcription complex [24]. A typical case would involve a regulatory protein which binds to promoter regions of the gene and also binds to other regulatory proteins. Some of these other proteins bind to enhancer sequences on the DNA while others do not bind DNA directly (but bind to DNA binding proteins) and this complex binds to the RNA polymerase which will then initiate transcription to produce the RNA. The cooperative interaction of these various proteins with the DNA, with one another, and with RNA polymerase increases the likelihood that an RNA polymerase will be bound and that the gene will be transcribed. Some elements which bind to the RNA polymerase also increase its processivity and thus whether or not a full length transcript is actually obtained.

Once the RNA is produced, it may require other processing which involves RNA binding proteins. For example, eukaryotic genes frequently contain introns which must be removed and this process requires RNA binding proteins. Other proteins and enzymes recognize specific sequences at the 3′-end of the RNA which will result in a poly(A) tail being added and other enzymes will add a 5′-cap structure. The processed mRNA is then translated in a process that involves various RNA binding proteins, including of course those of the ribosome. These processes are somewhat different in prokaryotes but here also, DNA and RNA binding proteins serve important roles.

This short discussion reveals some of the problems which can be anticipated in using DNA affinity chromatography to purify and study the components of such complexes. When cellular extracts are applied to DNA columns, even columns containing only a specific DNA sequence, a sizable number of proteins frequently bind and subsequently elute. Usually only one of these proteins is shown to bind that specific DNA sequence with high affinity and all other proteins which bind to the column are often referred to as "non-specific" DNA binding proteins. However, these other proteins may often be specific members of the transcription complex under study. The complex may simply contain proteins which do not bind to DNA directly but rather bind to other DNA binding members of the complex. Other components of the complex may bind to DNA directly but are simply less fastidious in the sequences they will bind. Indeed, since RNA

polymerase must be able to bind a variety of templates as well as to specific promoters, it could be considered a less fastidious member of the complex. The approaches that are taken to decrease this "non-specific" binding — the inclusion of a competitor DNA in the mobile phase or pre-adsorption on a heterogeneous DNA support — are usually effective at allowing the purification of the desired DNA binding protein. If protein purification is the primary goal, then this approach represents a significant improvement; however, it should also be recognized that other members of the complex may be discarded by these procedures. Thus, specific DNA sequence supports are not always absolutely selective for a specific DNA binding protein and neither should they be expected to be.

DNA repair, replication, recombination, transposition, and formation of the nucleosome structure of eukaryotes all involve DNA binding proteins which could also be described. However, the discussion provided is sufficient to show the importance of these proteins to our basic understanding of biological processes. Since some oncogenes and protooncogenes (*e.g.*, fos, jun, and myc) which are important to our understanding of cancer and cell proliferation code for DNA binding proteins (see refs. 63 and 64 for review), viruses often contain enhancer regions that bind host transcription factors which insure their effective replication (*e.g.*, NF-1 in adenovirus, NF-κB and SP-1 in HIV, etc.) and other pathogens often rely on modification of the cell's regulation at this level for their function, a knowledge of these processes is also of practical importance to our health and well being.

DNA affinity chromatography has played an important role in the purification and characterization of these proteins. A recent article [1] listed 37 applications of DNA supports to this area of research. Over fifty DNA binding proteins have been purified and partially characterized.

4. THE SOLID PHASE

For low-performance chromatography, celluloses, agaroses, and acrylamides are the typical choices. While many of these supports are limited to pressures less than about 5 bar, recently several new polymeric supports have become available with pressure limits which range from 50 to 300 bar which could be used with modern high-performance chromatographs. The author has the most experience with macroporous silicas.

While many coupling procedures and other protocols used for low-performance, soft-gel chromatography are adaptable to HPLC and *vice versa*, some caution may be necessary when adapting a reported procedure to a new support. We have found that some methods developed for silica are not necessarily applicable to some other supports. For example, we have described enzymatic synthesis of a polyadenylated 21-mer template sequence onto a $(dT)_{18}$-silica [45]. When these same procedures were used with the $(dT)_{12-18}$-cellulose available from Pharmacia, the template sequence was bound by the support but enzymatic, template-directed synthesis did not occur to any significant extent (unpublished data). We are not certain why synthesis did not occur but this result suggests that adapting procedures may not always be a simple alternative.

4.1. Choice of support

4.1.1. General

The usual considerations pertaining to chromatographic resolution apply. Soft gels (*e.g.*, agarose, acrylamides) typically have poor mass transfer characteristics which lead to inefficient washing and peak broadening upon elution. They have low-pressure limits which may impose limits on flow-rate or column geometry for certain applications. The soft gels also shrink and swell as the ionic strength (osmotic pressure) of the mobile phase is changed. The rigid supports such as silicas, controlled-pore glass, and some of the new polymeric supports often give better performance for most kinds of chromatography.

The supports which have been used successfully for the separation of either polynucleotides or polynucleotide binding proteins include silicas [45–49], controlled pore glass [65], latex [44], polystyrene [43], cellulose [6–18], agarose [1,8,

17,19–40], PTFE [41], and paramagnetic beads (which can be recovered using a magnet) [42].

4.1.2. Pore size

Most data suggest that pore sizes in the range 30–50 nm are appropriate for most applications. For oligomeric DNA (18-mer), silicas with pores in this range give the best combination of coupling efficiency and capacity [48]. This seems to result from two competing factors. (1) In a double helix, an 18-mer would have a length near 6 nm. Thus, small-pore-size silicas (5–10 nm pores) probably exclude much of oligomeric DNA from the pores which lowers capacity. (2) Higher-pore-size silicas have lower surface areas which lowers available capacity. For example, 30-nm-pore silica typically has a surface area which is near 100 m^2/g while in 400-nm-pore silica, this is reduced to about 10 m^2/g. Thus, very small or very large pores limit capacity.

Silica of 30 nm pore has also been used for the isolation of poly(A) mRNA from *Saccharomyces* [47] and 50-nm-pore controlled-pore glass containing $(dT)_{30}$ was shown to also have a high capacity for poly(A) mRNA [65] suggesting that pore sizes in this range are also useful for kilobase-size polynucleotides. Since most DNA binding proteins which have been characterized so far have molecular masses in the range 20 000–120 000 and proteins of this size would not be excluded by pores in the 30–50 nm range, this size pore should also be useful for the DNA affinity chromatography of these proteins. To date though, these proteins have only been purified using either non-porous DNA-celluloses or DNA-Sepharose 4B which has a somewhat larger effective pore size.

4.1.3. Bead size

For high-performance chromatography and high resolution, 5–10 μm beaded silicas perform well [45–49]. Larger bead diameters would allow higher flow-rates and lower pressures while the reverse is true for smaller diameter beads. However, it should be pointed out that for affinity chromatography, selectivity is usually quite high and so the usual criteria for a high resolution column (small bead size, high surface area and efficient mass transfer, long columns of small diameter, etc.) need not be strictly adhered to. Columns which are not optimized for these parameters may still give adequate performance.

4.1.4. Choice of column-bound DNA and column dimensions

The purpose of the chromatography has a large effect on these choices but in general small columns containing high loads of DNA are preferred. Since the columns can be used for polynucleotide separations or protein separations we will deal with these separately.

For separating polynucleotides, we have used two column sizes most frequently: 30 mm × 4.6 mm I.D. and 23 mm × 2 mm I.D. columns. The smaller of these columns [48] had capacities of about 1 nmol for $(dA)_{18}$ (about 5 μg). The larger columns had capacities in excess of 2 nmol for $(dA)_{18}$ although the maximum capacity was never measured [46]; for mRNA, capacities over 80 μg were measured [47].

Small columns have adequate capacity for most uses. For example, a 30 mm × 4.6 mm I.D. column would bind all of the poly(A) mRNA we typically isolate from 1 g of mouse skeletal muscle (unpublished data). This is sufficient high-quality mRNA for cDNA library construction or for about 50 reverse transcriptase/polymerase chain reaction amplifications of specific cDNAs.

There is also a very good reason to prefer small columns containing high loads of attached DNA — they allow rapid chromatography and high recovery. The rate of hybrid formation is a function of the concentration of the two DNA strands, the one on the support and the applied strand in the mobile phase. When one strand is in excess, the rate of hybridization is pseudo first order and is described by

$$k = 3 \times 10^5 \times L^{1/2}/N \ (M^{-1}\ s^{-1}) \quad (1)$$

where k is the pseudo first-order rate constant, L is the length of probe (here attached to a support) and N is the sequence complexity of the duplex formed in base pairs (for review, see refs. 66 and 67). The time dependence for hybridization is

$$t_{1/2} = \ln 2/kC \quad (2)$$

where $t_{1/2}$ is the half-time (s) and C is the concentration (in molar nucleotide base) of whichever strand is in excess. While this equation only strictly applies at 0.18 M Na^+ and at 25°C below the melting temperature of the DNA–DNA hybrid formed (see below), the optimum temperature is quite broad [48,68] and salt concentrations up to 1 M increase the rate by less than an order of magnitude. Thus although the equation applies only to a certain set of conditions, none of the calculations given here would be much changed by other conditions. The kinetics of hybridization of DNA or RNA tethered to a solid support is similar to that for hybridization free in solution [68], and thus these equations probably approximate the behavior expected for DNA affinity supports [48].

These equations state that the most rapid hybridization will occur with short DNA sequences present in high concentration on the support. For the amounts of $(dT)_{18}$ which have been coupled in small HPLC columns (23 mm × 2 mm I.D.), half-times for hybridization of $(dA)_{18}$ of about 20 ms can be calculated [48]. Using these small columns, we have shown that complete hybridization occurred at the highest flow-rate possible without exceeding the pressure limits of the support [48]. Using somewhat larger columns (30 mm × 4.6 mm I.D.) also containing high amounts of bound DNA, the purification of poly(A) mRNA in about 8 min has been demonstrated [47].

However, while speed and retention are at a maximum for columns heavily loaded with short DNA sequences, these are not the only considerations. Operating temperature places a lower limit on the length of the column-attached DNA. DNA sequences as short as 3–4 bases will form hybrids with column sequences at temperatures near the freezing point of water [46]; for columns which must be able to form stable hybrids at room temperature (see below), the column-attached DNA must be at least a 10-mer or greater. However, column selectivity usually dictates even longer sequences. The human genome has a complexity of about $3 \cdot 10^9$ base pairs; considering both strands, there are $6 \cdot 10^9$ bases. For a given sequence to occur randomly only once in DNA mixtures of this complexity, the sequence must be at least a 17-mer (*i.e.*, $4^{17} > 6 \cdot 10^9$). Thus, for example, to be reasonably assured that a column could select for one particular mRNA from a human cell line, the column must have a complementary DNA sequence at least 17-mer in length. Use of an 18-mer sequence would provide a suitable margin for error for most purposes. For an 18-mer, the rate constant for hybridization defined above would be $7.1 \cdot 10^4$ M^{-1} s^{-1}. If a 1-ml column were to be operated at 1 ml/min and be expected to retain greater than 97% of the mRNA applied to it which contained the correct sequence, it must contain sufficient 18-mer DNA so that the 1 min a sample spends in the column represents about five half-times of hybridization. Thus, the column must contain at least 0.8 nmol DNA per ml (0.8 μM) to meet these criteria. Since coupling of even larger amounts is feasible with a variety of low- and high-pressure supports, sufficient DNA coupling can usually be obtained for rapid chromatography.

The above considerations do in fact match the behavior we have observed with $(dT)_{18}$-silica. For columns containing high loads of $(dT)_{18}$, hybridization with applied samples (*e.g.*, $(dA)_{18}$) was shown to be quite rapid [48]. As shown in Fig. 7, retention was in fact maximal at hybridization temperatures about 25°C below the calculated hybrid melting temperature [48].

How rapidly DNA supports can be used in separations of DNA binding proteins is not known but the chromatography does not appear to be noticeably slow. The high affinity of many of these proteins for DNA (*e.g.*, $K_d = 0.1$ nM for adenovirus major late transcription factor [22], 0.01 nM for the estrogen receptor [23], etc.) could suggest that DNA binding is quite rapid. In the few cases where the rate constants have been measured, this appears to be the case. For example, the DNA binding rate constant for the adenovirus major late promoter transcription factor is $2.7 \cdot 10^7$ M^{-1} s^{-1} at 30°C [22]. The rate of binding of these proteins to DNA will be highly dependent upon the effective DNA concentration coupled to the support but should be quite rapid

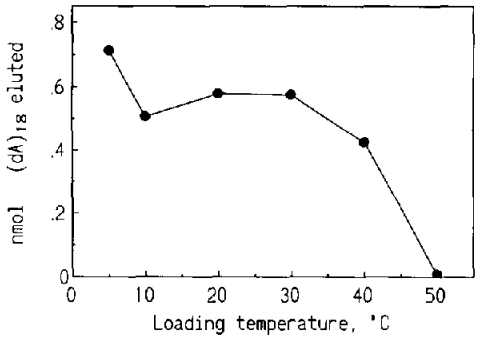

Fig. 7. Effect of loading temperature on column capacity. A 10-nm-pore $(dT)_{18}$ column (containing 11.8 nmol $(dT)_{18}$) equilibrated in TE500 buffer (10 m*M* Tris, 0.1 m*M* EDTA, 500 m*M* NaCl, pH 7.5) in a water bath at the various temperatures shown was loaded with 0.99 nmol of 5′-end-labeled $(dA)_{18}$ (23.1 cpm/pmol) in 10 μl of TE500. The column was then washed for 15 min under the loading conditions and eluted by transferring the column to a 65°C water bath. The flow-rate was 0.5 ml/min and 1-min fractions were collected. The fractions were counted for Cerenkov radiation to determine the amount of eluted $(dA)_{18}$. Under the conditions used, the melting temperature for the $(dT)_{18}$–$(dA)_{18}$ hybrid is 50°C; the results show that temperatures at least 25°C below the melting temperature give maximum retention by the column. Taken, with permission, from ref. 48.

for almost every conceivable case. For example, for the rate constant cited, a column containing 1 nmol DNA per ml should bind the protein with a half-time near 20 ms. Small columns containing high DNA loads should give the highest yields and most rapid chromatography.

However, while in the separation of polynucleotides, short (*e.g.*, 18-mer) DNA sequences are to be preferred, for protein separations longer DNA is to be preferred. In the purification of DNA binding proteins which have a specific DNA recognition sequence, it has been observed that retention and recovery during DNA affinity chromatography increases when multiple, tandem copies of the recognition sequence are used in column construction [7,19]. Usually 10–100 copies of the sequence are contained in these multimers. The procedures needed to construct such tandem multimers add some complexity to DNA column preparation. Many of these recognition sequences are of lengths in the range of 6–30 base pairs. To obtain a tandem multimer containing ten or more copies by direct chemical synthesis is impractical. Instead, what is usually done is to chemically prepare both complementary strands for a single-sequence copy such that the duplex will have a complementary 4–5 base 5′ or 3′ overhang at each end. These overhangs make the ends "sticky" so that they hybridize in solution to make non-covalent multimers which can then be ligated (see ref. 1 for an example of this procedure). The multimers are then purified and attached to a support for chromatography.

4.1.5. Column capacity and loading

For chromatography of DNA binding proteins, very little data are available on column capacity. Most of these proteins are present at only low concentrations in cells and, thus, large volumes of cellular extracts frequently must be processed. Under these conditions high flow-rates are more important than high capacity.

For the separation of mRNA and DNA, column capacities of over 1–2 nmol for small columns (23 mm × 2 mm I.D. or 30 mm × 4.6 mm I.D.) have been demonstrated. For kilobase-size mRNA, 88 μg capacities for 30 mm × 4.6 mm I.D. columns have been demonstrated [48]. The highest capacities would be expected for the supports made by direct synthesis on controlled-pore glass [65] or PTFE [41] which can be prepared with even higher amounts of attached DNA.

As has been noted above, as long as the amount of DNA coupled to the support is high, hybridization with applied DNA or RNA can be quite rapid and the sample DNA can simply be loaded into a sample loop and injected for HPLC or loaded onto the top of the column for low-pressure chromatography. With HPLC columns we have compared the amount of DNA retained following injection of DNA to that obtained following prolonged recirculation of the DNA sample and find little difference [48]. Usually when an applied DNA is not retained by a column, the speed of chromatography has not been the problem. Oligomers are usually dissolved in mobile phase and injected onto a column with no further treatment. However, longer DNA and RNA, because of inter- or intra-chain base

pairing, often must be denatured prior to loading onto the column if they are to be retained. For DNA, the most effective denaturation we have tried is alkaline denaturation. The DNA sample is made 0.2 *M* NaOH, 0.2 m*M* EDTA, incubated at room temperature for 5 min, neutralized with one tenth volume of 2 *M* ammonium acetate (pH 4.6), and precipitated with two volumes of ethanol. Immediately prior to injection, it is dissolved in mobile phase and injected. For RNA, this treatment should not be used because of the lability of RNA to strong alkali. Rather, for RNA we have found that heating to 95°C in water, followed by rapid cooling in an ice water slurry, and adding sufficient salt for column hybridization immediately prior to injection often improves retention by the columns. Whether or not denaturation is required for every sample is not known and should be discovered empirically.

4.2. Coupling chemistries

4.2.1. High-performance affinity chromatography

For coupling to macroporous silica, there have only been three kinds of coupling reported so far. They fall into two categories.

4.2.1.1. Chemical coupling

(1) Free radical copolymerization of a methacrylate-silica derivative with methacrylate or vinyl nucleic base derivatives [49]. This is not DNA coupling, but rather the preparation of a synthetic polymer containing nucleic acids.

(2) Coupling of 5′-aminoalkyl DNA derivatives to a NHS-ester silica [46–48].

We have also coupled 5′-aminoalkyl-$(dT)_{18}$ to glycidyloxypropyl-derivatized glass. Good coupling efficiency was found and the support hybridized $(dA)_{18}$ with relatively high capacity, but the chromatography has not been as well characterized as the other supports described (unpublished data).

4.2.1.2. Enzymatic synthesis

Template-directed enzymatic extension of a primer DNA-silica has been described [45].

4.2.2. Low-pressure affinity chromatography

Coupling to low-pressure chromatographic supports has been more varied. The first DNA-cellulose was prepared by carbodiimide activation of the 5′-phosphoryl of oligo(dT) to react with the cellulose hydroxyls to give an ester linkage [2]. The other methods can be conveniently grouped into three categories.

4.2.2.1. Direct synthesis on the support

Two reports fit this criterion. In one, $(dT)_{30}$ was synthesized directly on 50 nm pore, 125–177 μm diameter controlled-pore glass beads using an Applied Biosystems DNA synthesizer. After synthesis, instead of deprotecting the DNA in the usual way with concentrated ammonium hydroxide (which also cleaves the oligonucleotide from the support), deprotection was with a thiophenol–dioxane–triethylamine reagent which deblocked the phosphoryl groups while leaving the oligonucleotide attached. The resulting support was shown to have a high capacity for the purification of poly(A) mRNA [65]. This clever procedure unfortunately is limited to homooligomeric dT (or dU) support synthesis; other nucleotides (A, C, and G) would contain additional protecting groups which would require harsher treatments to remove which would also cleave the DNA from the support.

A second method [41] apparently solves this problem. The support in this case was PTFE fibers which contain a hydroxyl group at the end of an aliphatic spacer. Chemical DNA synthesis can be characterized as a sequence of steps in which an activated nucleotide phosphate reacts with the 5′-hydroxyl of the previously added nucleotide to give a phosphodiester linkage. Conditions used to deprotect and cleave DNA from supports are of course gentle enough to preserve these phosphodiesters. So that DNA can be cleaved from the support, the linkage of the first (3′) nucleotide back to the support is usually a more labile linkage. For synthesis of an HPLC support, reacting the first nucleotide phosphoramidite with a hydroxyl-containing support results in a stable phosphodiester linkage which will survive deprotection chemistry intact, leaving the

DNA attached. Since this deprotection chemistry involves prolonged exposure to concentrated ammonium hydroxide, an inert support such as PTFE is a good choice.

Using this PTFE fiber-based method, a specific 37-mer DNA sequence was synthesized using an Applied Biosystems DNA synthesizer, was deblocked and hybridized to the complementary strand to make a dsDNA support, and the fibers were used to successfully purify a DNA binding protein which specifically recognizes this DNA sequence [41]. The advantages of this method are the high amounts of DNA which can be coupled, the single (3′-end) point of attachment with no modification of nucleotide bases, and the simplicity of preparing the support on existing DNA synthesizers. The limitations are that the method would be ineffective for applications requiring very long DNA sequences, heterogeneous dsDNA sequences, or for coupling a DNA isolated from biological source. However, for those uses where applicable, the method has significant advantages.

4.2.2.2. Chemical coupling

Cyanogen bromide (CNBr). By far, the largest number of reports have used DNA coupling to CNBr-activated Sepharose and other agaroses [1, 8,17,19–31,33,35–40]. The method can be traced back to 1971 [20] and the most widely used method has been recently reviewed [1]. dsDNA of a specific sequence is coupled, presumably by way of the nucleophilic bases (*i.e.*, dA, dC, dG). Since these bases are also critical to the biological affinity upon which the chromatography relies, this coupling has little to recommend it except its simplicity and the undisputed fact of its success in purifying DNA binding proteins. The coupling procedure itself is simple [1,21]. Sepharose can either be CNBr-activated in the laboratory or purchased in an activated form. The support, in mildly alkaline buffers (*e.g.*, pH 8), is then mixed with the DNA and allowed to react, typically overnight. Excess reactive groups are then blocked (*e.g.*, with aminoethanol) and the support is ready for use.

Amide. An ion-exchange silica, Macrosphere WCX (Alltech), contains a carboxylate which, upon reaction with a carbodiimide and N-hydroxysuccinimide (NHS) becomes an activated NHS-ester silica [50]. This activated ester reacts preferentially with a 5′-aminoalkyl moiety introduced into synthetic DNA to produce a stable amide linkage of the support to the DNA [46,48]. This coupling chemistry will also couple DNA by way of nucleotide base nucleophiles, however, in ssDNA reaction with the 5′-amino is preferred nearly 70-fold over reaction with the nucleotide bases and in dsDNA the bases are protected and little reaction occurs [45].

Coupling of single-stranded poly(A) by way of the nucleotide bases to BioRad's AffiGel 10, using similar chemistry, has been demonstrated and used successfully [52]. Reardon [34] has used AffiGel 15 (again similar chemistry) to couple a partially single-stranded, specific-sequence DNA to a support after first introducing a 5′-aminohexyl moiety into the DNA using a procedure originally developed by Chollet and Kawashima [69].

Thioether. Synthetic DNA containing a 5′-thiol was hybridized with its complementary strand and the dsDNA has been coupled to both Tresyl- and epoxy-activated Sepharose [32]. In either case, the coupling chemistry would yield a thioether linkage at the 5′-end. Whether or not reaction also occurs by way of the nucleotide bases was not determined. The resulting support was used for a simple purification protocol for *Eco* RI restriction endonuclease.

4.2.2.3. Other coupling procedures

Two other widely used coupling procedures fit none of the above categories.

Drying onto cellulose. This method, described by Alberts and Herrick [6], has been widely used. The chemical basis of this form of coupling is unknown but the procedure is widely used and works well. Procedures for making columns from dsDNA or denatured dsDNA ("ssDNA") are available [6]. The procedure is quite simple. DNA solutions are simply mixed with cellulose powder, allowed to air dry, and then lyophilized. Once a slurry is reconstituted, most of the DNA remains

bound the cellulose as long as the DNA-cellulose is not heated above about 50°C. The DNA is not covalently attached to the cellulose, but rather dissociates very slowly over time; *e.g.*, the half-time for DNA release is about 400 h at 37°C [6].

As described above, many kinds of DNA binding proteins must bind to DNA regardless of its sequence (*e.g.*, DNA replicating enzymes, recombination proteins, etc.) and, in the purification of any DNA binding protein, these are either the fraction being sought [8–11,31,33] or which must be removed [8,14,17,27] during purification. Thus, columns which contain DNA of heterogeneous sequence (*e.g.*, genomic DNA) are clearly needed in the purification of DNA binding proteins. Because the DNA-cellulose procedure is well adapted to preparing such columns, columns made in this way are widely used in DNA binding protein isolation. For DNA binding proteins which bind a specific recognition sequence, heterogeneous DNA-cellulose columns are often used in conjunction with specific sequence DNA columns [8,14,17,27] (prepared by drying on cellulose [7,16] or, more commonly, attached covalently to CNBr-activated Sepharose [1,19] or in other ways) to recover a specific DNA binding protein of interest.

Biotinylated DNAs. Another common coupling procedure is to use the biotin–avidin interaction to produce DNA supports. Biotin binds tightly ($K_d = 10^{-15}$ *M*) to avidin, a protein isolated from egg whites, or to streptavidin, a protein isolated from *Streptomyces*. Biotin can be incorporated into DNA either enzymatically [12,22,26,28,42] by using biotinylated derivatives of nucleoside triphosphates or chemically (or photochemically) [35,43] with reactive derivatives of biotin. The enzymatic incorporation has been more widely used. Biotinylated nucleoside triphosphates (*e.g.*, Biotin-7-dATP, Biotin-11-dUTP, etc.) are available commercially (*e.g.*, Bethesda Research Laboratory, Clontech, etc.) and can be incorporated into DNA by many DNA synthesizing enzymes (*e.g.*, Klenow large fragment DNA polymerase I, T4 polymerase, avian myeloblastosis virus reverse transcriptase, etc.) [26]. Using nick translation, multiple biotinylated nucleotides can be incorporated throughout a sequence, however, the most common approach for chromatography has been to enzymatically incorporate one or more biotin nucleotides at the 3′-end of one strand of the DNA [12,22,26,28,42]. This is accomplished by digesting the DNA with a restriction endonuclease which produces a 5′ overhang (*e.g.*, *Eco* RI, *Hind* III, *Sal* I, etc.) and filling in the 3′ recess with DNA polymerase using an appropriate biotinylated dNTP. Reagents are also available for incorporating biotin at the 5′-end during chemical oligonucleotide synthesis (available from Pharmacia, Applied Biosystems, and most other reagent suppliers; more recently, a biotin-phosphoramidite derivative has become available from New England Nuclear-Du Pont).

Once DNA has been biotinylated, it can be attached to any avidin, streptavidin, or biotin support desired. [Avidin or streptavidin have more than one biotin binding site (*e.g.*, chicken egg white avidin has four) and can serve as a bridge between the biotinylated DNA and biotinylated support in the latter case.] Many of these supports are commercially available or can be easily prepared. One particularly interesting support is streptavidin attached to small paramagnetic beads [commercially available as streptavidin-Dynabeads from Dynal A/S (Oslo, Norway) or streptavidin-MagneSphere from Promega (Madison, WI, USA)] which have been used for the isolation of at least one DNA binding protein [42]. Promega also sells these beads in a kit which contains biotinylated oligo(dT) and provide a protocol for using the kit for poly(A) mRNA isolation.

There are several potential problems with the biotinylated DNA-(strept)avidin support approach to DNA binding protein chromatography. Extremes of temperature or denaturing solvents must be avoided during chromatography and, perhaps more to the point, during column cleaning and regeneration between uses. Crude fractions which may be rich in proteases should be avoided. The additional biotin–streptavidin interactions may lead to additional proteins being retained by the columns; *e.g.*, proteins with a

biocytin prosthetic group or proteins which bind biotin or (strept)avidin. It has been reported in the isolation of one DNA binding protein that actin, which contaminated the DNA binding proteins obtained, resulted from actin binding to the streptavidin during chromatography [35].

4.2.3. Determining the amount coupled

The amount of DNA coupled can be estimated by several means. The easiest is to simply use the high absorption of DNA at 260 nm to quantify the amount added during coupling and subtracting the amount recovered in all of the washes of the coupled support. If done carefully with rigorous washing, this procedure can be quite accurate [48]. Alternatively, the DNA can be radiolabeled (see below) and the amount recovered on the support quantified by scintillation counting. If this procedure is used, the specific activity of labeling should be kept relatively low to prevent damage of the DNA support. Except for those supports containing very low amounts of DNA, hydrolysis of the support using $Mg(NO_3)_2$ and ashing followed by phosphate determination yields reliable estimates of DNA bound for either silica-based [48] or agarose-based [21] supports.

These methods all give the amount of DNA bound by the support but do not necessarily give the amount able to participate in the chromatography. We have found that the amount of ssDNA attached to silica may be over twenty-fold higher than the actual capacity of the support measured by hybridization [48]. Whenever possible, the actual capacity should be measured but this may not always be feasible especially for columns routinely used for DNA binding protein isolation.

5. FACTORS INFLUENCING HYBRID FORMATION

Base composition, temperature, ionic strength, and denaturants all effect hybridization, and empirical relationships describing the effect of each of these on hybrid melting temperatures have been derived. For oligonucleotides from 11–23 bases long in 0.9–1.0 *M* Na^+, the melting temperature of DNA–DNA hybrids (T_d) is described by [66,68]

$$T_d = 2\ (\#A + T) + 4\ (\#G + C) \quad (3)$$

where $(\#A + T)$ is the number of adenine and thymidine bases. This equation tends to overestimate melting temperatures for long hybrids. For longer polynucleotides (greater than 50 bases), the three different kinds of hybrids possible (DNA–DNA, DNA–RNA, RNA–RNA) each has a derived empirical relationship [67,68].

The equation we have found most useful for predicting DNA–DNA hybrid melting temperatures in our chromatography experiments is the one given by Sambrook *et al.* [70]

$$T_m = 81.5°C + 16.6 \log_{10} [Na^+] + 0.41\ (\%\ G + C) - 0.63\ (\%\ \text{formamide}) - 600/L \quad (4)$$

where T_m is the melting temperature of the hybrid (synonymous with T_d), $[Na^+]$ is the molar Na^+ concentration, and L is the length of the hybrid formed (in base pairs) and should be in the range of 14–70 base pairs.

Some cautions must apply to the use of these equations. Fo example, the last equation gives reasonable predictions in our experiments for salt concentrations below about 0.5 *M*. An excellent discussion of T_m, rates of hybridization, and the effect of experimental conditions is given by Meinkoth and Wahl [67]. These equations allow one to calculate conditions under which a column can be safely washed and give a lower limit for where elution will occur; usually elution actually occurs several degrees above this lower limit. Since most chromatography has no equations to predict elution behavior, these equations are an important and rare resource.

These equatons are useful for all of the forms of DNA affinity chromatography. For DNA binding protein chromatography, they give the limits under which a duplex column can be maintained. Theoretically, at least it should be possible to use strand separation to effect protein elution but the author is unaware of any case where this has actually been used for elution.

In oligonucleotide [46], DNA [45] and mRNA [47] separations by DNA affinity HPLC, we have

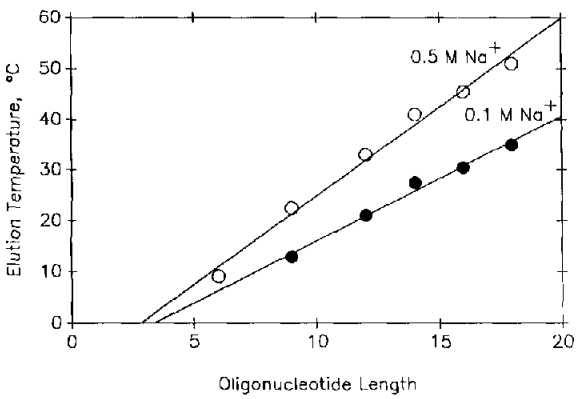

Fig. 8. Salt, temperature, and chain length dependence of DNA-silica elution. Shown are the temperatures at which oligoadenylates from lengths 6 to 18 eluted from a $(dT)_{18}$ column using mobile phases which contained either 0.5 *M* Na^+ (open circles) or 0.1 *M* Na^+ (closed circles). The results show that increasing chain length by one nucleotide causes a 2.5–3.5°C increase in elution temperature at 0.1–0.5 *M* salt, respectively. Eqn. 4 given elsewhere in this review would predict a 2.5°C increase and, for example, would predict that $(dA)_{18}$ would elute at 43°C (in 0.5 *M* salt) instead of the 50°C found. This figure is taken, with permission, from ref. 46.

found a reasonably good correspondence between predicted elution conditions and those actually found. For example, the effect of [NaCl] on elution temperature shown in Fig. 8 matches reasonably well what would be estimated by the relevant empirical equations.

6. CHROMATOGRAPHY AND ELUTION CONDITIONS

6.1. Polynucleotide separations

For separations involving hybrid formation between an ssDNA support and an applied polynucleotide sample (*i.e.*, in the fractionation of oligomers, poly(A) mRNA, etc.), the equations given above allow retention and elution conditions to be predicted. For this kind of chromatography, we have found salt concentration and temperature to be the most useful variables to change during chromatography. Formamide, even freshly deionized, often has significant absorption at 260 nm which interferes with DNA or RNA detection. For very long DNA or RNA hybrids, melting temperature (T_m) can become quite high in high salt mobile phases and so, frequently, a combination of increasing temperature and decreasing salt concentration is the most useful strategy for elution.

For polynucleotide isolation from cells, it is important to protect the column from the abundant nucleases in cell extracts. DNA supports can be protected effectively by adding EDTA to the mobile phase (see above). However, if the RNA is being isolated, protecting the column is not nearly as difficult as protecting the sample from degradation by RNases.

During the purification of mRNA [47], we experienced many of these problems associated with RNases. RNases can collect on surfaces within the chromatograph and cause sample degradation during chromatography. We found that by cleaning the chromatograph regularly, this problem could be averted. The column is first removed and replaced by a union. The chromatograph is then cleaned by pumping 10% nitric acid for about 30 min through all non-column parts, including the detector and fraction collector. The chromatograph is then rinsed with diethylpyrocarbonate (DEPC, an RNase inhibitor)-treated, autoclaved water and can then be considered (relatively) RNase free. For work with RNA, all aqueous mobile phases were also prepared with DEPC-treated, sterile water [47]. Perhaps the best treatment of all though is to avoid applying crude fractions to the chromatograph by initially partially purifying the RNA. For *Saccharomyces*, an initial purification using phenol–chloroform extraction, LiCl precipitation, and then ethanol precipitation of the RNA [71] was effective in depleting the samples of nucleases prior to chromatography.

6.2. Polynucleotide binding proteins

There are several ways in which a DNA binding protein could be eluted from a DNA column (*e.g.*, inducing DNA strand separation to elute dsDNA binding proteins, adding the appropriate DNA to the mobile phase, denaturing either the protein or the DNA, etc.). Except for one case where elution

of lac repressor protein was accomplished with the addition of IPTG or by restricting the DNA support [28], these proteins are almost always eluted using an increasing salt gradient; most elute in the range 0.2–2 *M* KCl or NaCl.

Ideally, a column could be prepared with the specific polynucleotide sequence recognized by the protein of interest and this would be the only protein bound by the column. Past experience with the chromatography of DNA binding proteins has not always matched this expectation. Columns containing specific DNA sequences typically bind other proteins in addition to the one of interest as has been discussed above. Three strategies have been developed for making the chromatography more selective. One strategy uses two DNA columns: one containing only the specific DNA sequence and another "non-specific" column containing either a heterogeneous mixture of DNAs [7,8,17,27] (*e.g.*, fragmented calf thymus DNA) or DNA known to lack the specific sequence [25]. A sample is applied to the "non-specific" column first and the specific DNA sequence column last. Another strategy has been to simply repeat chromatography on the same DNA column. While repeated chromatography often results in only small improvements in purity in other situations, it often results in much greater purity for DNA binding proteins [24,25,30,40]. A final strategy has been to add a competing DNA such as poly(dI,dC) to the sample prior to chromatography on a single, specific-sequence column [1,8,22,28–30,35,38,40]. All of these strategies have been successful in certain applications, but the latter has certain advantages which make it preferable including the need to prepare only one column and empirical methods for determining how much of the competing DNA to add without adversely affecting yield [1].

However, whether the use of competitor DNA (or other approaches to preventing "non-specific" binding) is necessary in many cases is questionable. Many DNA binding proteins have been purified using none of these precautions [9,10,12, 26,31,32,34,39,41] and some of these are quite simple procedures yielding highly purified proteins. Thus, although these precautions may not always be necessary, they may make purification easier in some cases.

Only seldom has DNA affinity chromatography alone been sufficient to purify a protein to apparent homogeneity [24,27,33,34]; other forms of separation must be relied upon during purification. In this regard, we should mention three other forms of chromatography which appear commonly in these purifications. Phosphocellulose ion-exchange chromatography is often used and it is interesting to speculate that it may additionally function here somewhat like an affinity chromatography support. DNA binding proteins, at least those which bind dsDNA, have many interactions with the sugar phosphate backbone of DNA and interactions with the sugar phosphates of phosphocellulose may, to some extent, mimic these interactions. Also heparin-Sepharose is used frequently in these separations. Recently, Jackson and Tijan [38] have used the observation that some transcription factors are post-translationally modified with N-acetylglucosamine (NAG) to advantage; they have used succinylated wheat germ agglutinin-agarose, which binds NAG, to facilitate the purification of SP1.

7. DETECTION

7.1. Ultraviolet absorption

The purine and pyrimidine bases which make up DNA and RNA absorb strongly at 260 nm with effective molar absorptivities per base in the range 7050–15 200 M^{-1} cm^{-1} [66]. This yields quite high absorptivities for even short oligomers. For example, the calculated [66] molar absorptivity for $(dT)_{18}$ is $1.5 \cdot 10^5$ M^{-1} cm^{-1}.

This high absorbance has led to the use of "A_{260} units" as a measure of polynucleotide concentration which may not be known to all chromatographers. One A_{260} unit is the amount of polynucleotide which, when dissolved in a volume of 1 ml, will give an absorbance of 1 at 260 nm. Conversion factors useful for long polynucleotides are that 1 unit of dsDNA is about 50 μg, for ssDNA or RNA 1 unit is about 40 μg.

Protein elution is typically detected at 280 nm

using a flow detector or is detected post-column (see below).

7.2. Radioisotopes

Several labeling reactions are available for incorporating radioisotopes into DNA or RNA. Synthetic, oligomeric DNA has a 5′-hydroxyl which can be labeled with γ-^{32}P-ATP using the T4 polynucleotide kinase reaction. Since γ-^{32}P-ATP is available in specific activities in excess of 5000 Ci/mmol and incorporations of over 50% can be obtained, detection of attomoles or less is clearly feasible. For DNA or RNA which has a terminal 5′-phosphate, labeling by this enzyme will also occur by an exchange reaction if ADP is included in the reaction mixture, though often labeling is less efficient. The 3′-end of DNA or RNA can be labeled using α-[^{32}P]dNTP or α-[^{35}S](thiophosphoryl)-dNTP using terminal deoxynucleotidyl transferase. This enzyme will attach multiple (dN) residues in a poly(dN) tail of indeterminate length. If attachment of a single label is desired, the dideoxy analogues of dNTPs or cordycepin triphosphate can be used. These and many other ways of labeling DNA and RNA can be found in most cloning manuals or in application notes provided by radiochemical and enzyme suppliers.

7.3. Post-column detection

Polynucleotide detection

In the gel electrophoresis, DNA and RNA are typically stained with the fluorescent compound ethidium bromide; detection of several tens of nanograms of DNA is feasible. Ethidium bromide in aqueous solution has a low fluorescence which is enhanced when it intercalates between the bases of DNA or RNA. This suggests that mixing polynucleotides postcolumn with ethidium bromide might provide an effective detection scheme. However, ethidium bromide is a potent mutagen which must be used with caution and disposed of properly.

7.3.1. Oligonucleotide probes

Another strategy for the detection of eluted DNAs or RNAs would be to simply spot the eluent onto nitrocellulose (or other appropriate media) and probe it with an appropriate complementary (antisense), radiolabeled oligonucleotide. Procedures which could be adapted to this purpose can be found in most cloning manuals. Spotting DNA binding proteins onto nitrocellulose and probing with a radiolabeled oligonucleotide would also provide a sensitive method of detection, but in practice a more discriminating assay is used which is described below.

7.3.2. Gel mobility shift assay

A gel mobility shift assay [72] is typically used to detect DNA binding proteins. In this assay, a radiolabeled (see above) fragment of DNA known to contain the regulatory sequence is applied to (non-denaturing) agarose or acrylamide gel electrophoresis. The DNA fragment is also mixed with a protein fraction thought to contain the DNA binding protein of interest and this is also applied to the gel. If the protein is present, it will bind to the DNA and retard its migration during electrophoresis and in that way be detected by autoradiography. Preparative gel mobility shift electrophoresis has also been used to purify NF-1, a DNA binding protein [73].

8. POTENTIAL USES AND FUTURE DIRECTION

The potential widespread usage of these supports in the purification of DNA, RNA, and their binding proteins has been partially realized as described above. Given the complexity of transcriptional regulation, recombination, etc., and the success of the affinity chromatography approach, this kind of chromatography will undoubtedly be used further. However, there are also several other potential uses which have not yet been realized. Some of these will be speculated on here.

8.1. Antisense DNA and RNA

In prokaryotes, antisense RNA serves important regulatory roles. For example, antisense RNA sequences serve roles in regulating plasmid copy number in host bacteria. There are other cases in prokaryotes where a short, complementary (*i.e.*, antisense) RNA binds to existing mRNA and prevents its translation (see ref. 74 for review). Whether or not antisense also serves similar roles in eukaryotes is less certain. DNA or RNA affinity chromatography could be used to analyze for and isolate such antisense sequences.

Whether or not antisense RNA serves a physiological role in eukaryotes, artificially introduced antisense DNA and RNA sequences have proved to be important probes in cell and developmental biology research (see ref. 75 for review). There is also considerable pharmacological interest in the potential uses of antisense. In cultured cells, viral replication can be prevented by adding an antisense oligodeoxynucleotide which targets (*i.e.*, is complementary to) a viral DNA or RNA sequence and prevents its successful production or utilization. Oligodeoxynucleotides when simply added to culture media are taken up by cells and are effective in preventing viral replication [76–80]. In addition to the potential antiviral uses of antisense, there are other potential uses. Many pathological conditions have a known or suspected biochemical lesion. Often inhibition of a specific enzyme (or inhibition of a specific receptor binding its ligand) is sufficient to ameliorate a pathological condition. Much of the current pharmacology involves the use of drugs which bind to a receptor or enzyme and prevent normal function. For example, β-adrenergic receptor antagonists (*i.e.*, β-blockers) are useful in the treatment of heart disease. The discovery of these specific chemical inhibitors has been a slow process. Antisense sequences may provide another class of pharmaceutical agent; in fact, biotechnology companies whose purpose is to investigate the pharmaceutical uses of antisense DNA have already been formed (*e.g.*, ISIS Pharmaceuticals, Carlsbad, CA, USA). The concept is that if a DNA (or RNA) sequence which is complementary (antisense) to the mRNA necessary for virus, enzyme, or receptors production is delivered to the cell, it should block (or partially block) translation of protein and give the desired inhibition at the level of protein synthesis. The feasibility of blocking translation using antisense RNA or DNA has already been amply shown [81–84]. Finally, transformed cells which serve as models for cancer can be restored to more normal cell division by using antisense DNA or RNA directed against protooncogene sequences [85,86]. Thus, if effective ways can be discovered to deliver antisense DNA and RNA to cells in a clinical setting, an important new class of pharmaceuticals may result with potential antiviral, anticancer, and other pharmacologically important uses. For use in humans, such antisense DNA or RNA would have to be of the highest purity. DNA affinity chromatography has the potential for such sequence-specific purification of antisense sequences. It may also potentially serve a role in production of DNA and RNA for antisense and other uses (see below).

8.2. DNA sequencing

Other potential uses of immobilized DNA can best be summarized by analogy to the uses of immobilized enzymes and proteins in biochemistry. Modern protein sequence is usually carried out with the protein (or peptide) immobilized on a solid surface [87]. This allows reagents to be added for the sequential removal of amino acids without the loss of protein/peptide and also allows for the automation of the necessary chemistry. DNA sequencing is carried out in a very different way, but there is little doubt that automation of these steps is essential for large sequencing projects such as the human genome project. By immobilizing DNA to a solid support, this automation may become easier. In the dideoxy method of DNA sequencing [88], fragments of DNA are produced by random termination of template-directed DNA synthesis. These fragments are then resolved by high-voltage electrophoresis on thin acrylamide gels which are easily overloaded. Each sequencing gel is only capable of resolving

DNA fragments up to about 400 base pairs in length. By producing these fragments on an ssDNA template tethered to a solid support, the fragments could be selectively eluted so that excess primer, radiolabels, etc. are removed and only fragments less than 400 base pairs are recovered in high concentration for electrophoresis. By applying only those fragments resolvable by electrophoresis, less DNA can be applied to the gel making overloading less likely. Prior to the development of enzymatic synthesis of DNA-silica [45], there was no way to produce immobilized ssDNA templates that was easy enough to perform to justify its use in DNA sequencing, but that situation may now have changed.

8.3. DNA and RNA production

Immobilized biochemicals are also used for production. For example, immobilized enzymes are used industrially for the production of needed biochemicals, sometimes on a large scale (*e.g.*, the enzymatic production of corn sweetener). DNA and RNA are not usually needed in large amounts (except perhaps for pharmaceutical antisense DNA, see above) but their production is of no less interest. Single-stranded DNA or RNA is often needed as a probe, for use in DNA sequencing, and for other uses. Single-stranded DNA can be produced by asymmetric PCR or from single-stranded viruses or phagemids. Plasmids containing viral RNA polymerase (*e.g.*, T7, SP6, etc.) promoters suitably placed for efficient ssRNA production are available. However, DNA supports also would potentially allow the production of these single-stranded polynucleotides on a reusable template and facilitate their purification. For example, published procedures allow DNA-silica to be produced containing virtually any DNA sequence [45]. The methods used attach a single strand of DNA covalently to the silica by a linkage to its 5′-terminus. Once this DNA-silica is made, it can be used along with primer oligonucleotide, as a template for the enzymatic synthesis of the duplex strand [45]. Once the strand is produced, the support can be easily washed to remove byproducts and substrates used during synthesis and eluted under conditions which should yield highly purified ssDNA. By incorporating viral RNA polymerase promoters (*e.g.*, T7, SP6, etc.) into the immobilized DNA sequence, the support could be used for RNA production. By using radiolabeled nucleotide triphosphates during enzymatic synthesis, single-stranded DNA or RNA probes of high specific activity in highly purified form could be obtained for research or clinical testing.

8.4. Library screening

DNA supports also have the potential to make DNA library screening more productive. For example, in screening cDNA libraries a quite common occurrence is that a partial cDNA, one that contains only a portion of the sequence of interest, is obtained. The usual approach is to then radiolabel this partial cDNA and use it as a probe to rescreen the library or to screen a new library searching for other partial or (with luck) a complete cDNA. This is often a very time-consuming process, especially when long sequences are being sought. Once a cDNA is obtained, it is often desirable to then use it as a probe to rescreen a genomic DNA library to obtain further information (*i.e.*, about upstream regulatory elements or the position and number of introns, etc.). A partial cDNA could also be used to prepare a cDNA affinity chromatography support. The uses are in many ways analogous to the use of a cDNA as a probe. That is, the column could be used to selectively purify from library DNA other cDNAs containing parts or all of this column's cDNA sequence and in that way enrich those sequences most likely to contain other partial or full-length cDNAs. The column would also be useful for screening genomic libraries. If cDNA library screening is not totally successful, the column could be used to purify mRNA coding for the sequence of interest to prepare a new cDNA library. This new library, being highly enriched for the sequence of interest, may more rapidly yield the full-length sequence.

9. CONCLUSIONS

The potential uses outlined here have not all been realized and some may not be practical. However, this speculation does suggest that DNA supports may have other uses in addition to the chromatography reviewed here. Some of this potential may be as important as the current usage.

The affinity chromatography alone is powerful enough to justify continued development. Fractionation of polynucleotides, especially mRNA, is a mainstay method of the biomedical sciences. The investigations of replication, transcription, translation, transposition, recombination, etc. are still in their infancy and have developed only as the ability to resolve the polynucleotide binding proteins improved. DNA affinity chromatography provides the needed technique for purifying these proteins in amounts sufficient for their characterization. The methods currently in use employ primarily cellulose and agarose supports and salt gradient elution. Higher-resolution supports would probably facilitate the fractionation and make for less arduous purification procedures.

Polynucleotide affinity chromatography, like most affinity methods, is already a high-resolution technique. By adapting these procedures to HPLC, improvements in speed, detection sensitivity, and resolution can be expected. Perhaps more importantly, these new technologies also provide novel materials which can be applied in other ways to solve research and manufacturing problems.

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