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Salt-induced immobilizations of DNA oligonucleotides on an epoxide-activated high-performance liquid chromatographic affinity support

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Abstract

Synthetic oligonucleotides, possessing a recognition sequence for the transcription factor NF-κB, were immobilized onto an epoxide-activated hydroxyethylmethacrylate HPLC affinity support in the presence of high concentrations of potassium phosphate. The extent of immobilization increased with salt concentration in a manner analogous to that which has been reported for salt-induced immobilizations of proteins. High immobilization efficiencies were achieved, and at 2.7 *M* potassium phosphate, 85–90% of the DNA initially present in the reaction mixture was immobilized. Reactions were examined for double stranded DNA, one strand of which was modified with a 5′-mercaptohexyl spacer arm, and for each of the strands comprising the duplex. For double stranded immobilizations, about 85% of the non-modified strand (the d22 strand) was released from the support under melting conditions, suggesting that d22 exhibited low reactivity when organized as the duplex. For immobilizations of single stranded DNA, mild acid hydrolysis of the products was used to provide information concerning the mode of attachment. For reactions of the d22 strand alone, only about 60% each of guanine and adenine were recovered from the immobilized oligonucleotide following mild acid hydrolysis. This suggests that when d22 is immobilized as the single strand, significant attachment occurs through the purine bases, in contrast with the low reactivity exhibited by d22 in the duplex. Purified p50 protein, the DNA binding element of NF-κB, and nuclear extracts from phorbol ester-stimulated HeLa cells were injected onto a column packed with the double stranded product. In both cases p50 was retained on the column and was recovered upon elution with a salt gradient.

Keywords: Immobilized oligonucleotides; Affinity adsorbents; Stationary phases, LC; DNA; Oligonucleotides

1. Introduction

Chromatographic supports for the affinity extraction of DNA binding proteins, or complementary polynucleotides, employ both covalently and non-covalently immobilized DNA. Chemistries for the covalent attachment of DNA have been described for reactions occurring through the DNA bases and from end point attachment of DNA molecules to the support. Examples of base attachment occur in reactions of DNA with polysaccharide supports activated with cyanogen bromide or diazotized aromatic amines [1,2]. Cyanogen bromide activated supports in particular have been used in the purification of many transcription factors, more than 20 examples of which are cited in Ref. [3].

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Although these kinds of supports have been successfully used in many applications, the suspicion that covalent attachment through the bases could interfere with DNA binding specificity has led to interest in site-specific immobilization of DNA. For example, Solomon et al. have used as a starting point $(T)_{18}$-derivatized silica (7 μm, 300 Å) hybridized to an oligonucleotide through a $(dA)_{18}$ tail. The immobilized oligonucleotide served as a template for DNA polymerase catalyzed synthesis of the complementary strand [4]. Attachment of DNA directly through the 5′-phosphate has been described for carbodiimide mediated reactions with cellulose and Sephadex [2,5,6]. Immobilizations of 5′-aminoalkyl modified oligonucleotides in the presence of carbodiimides have also been reported for reactions with carboxyl-derivatized supports, either directly, or through an N-hydroxylsuccinimide intermediate [7]. Supports for these immobilizations included carboxyl-derivatized Sephacryl, controlled-pore glass, crosslinked polystyrene and silica.

Immobilization of oligonucleotides modified at the 5′-phosphate with aminoalkyl or mercaptoalkyl groups provides a dual advantage: it permits the introduction of a relatively reactive terminal nucleophile possessing a higher reactivity than the nucleotide bases; in addition, the alkyl chain acts as a spacer arm for displacement of the DNA affinity ligand away from the surface of the support. Using this approach, 5′-mercaptopropyl derivatives of double stranded eicosomeric and dodecameric oligonucleotides were immobilized, respectively, on epoxide and tresyl activated Sepharoses [8]. The immobilization efficiencies reported for these reactions were 15–20% of the initial oligonucleotide when carried out in a large excess of epoxide or tresyl concentration.

Epoxide activated chromatographic supports have been widely used for many different applications involving affinity separations. For those applications requiring immobilized proteins, the efficiency of the immobilization is dramatically enhanced when performed in relatively high concentrations of salt [9–12]. This probably results from salt-induced partition of the protein along the surface of the support, in proximity with the epoxide groups, which would have the effect of promoting the reaction [12]. Reactions performed under these conditions generally present >90% yields based on initial protein in the reaction. In the present study, the application of high-salt media was examined for the immobilization of oligonucleotides on an epoxide modified, crosslinked hydroxyethylmethacrylate (HEMA) HPLC support. Immobilizations were performed using double stranded DNA, one strand of which was modified with a terminal mercaptohexyl spacer arm, and with each of the individual strands. The oligonucleotide selected for this study includes a recognition site for the transcription factor NF-κB.

2. Experimental

2.1. Reagents

Nuclease P1 from *Penicillium citrinum* was obtained from Boehringer Mannheim (Indianapolis, IN, USA). Nonidet P40 was from BDH Chemicals (Poole, UK). Dithiothreitol was from Sigma (St. Louis, MO, USA). Mononucleotides, nucleosides, nucleoside bases and glycerol were from Aldrich (St. Louis, MO, USA). Rabbit IgG antibodies for NF-κB subunit p50 and nuclear extracts were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alkaline phosphatase-conjugated goat anti-rabbit antibodies were from Perry and Kirkegaard Laboratories (Gaithersburg, TN, USA). BCIP (5-bromo-4-chloro-3-indolyl phosphate), NBT (Nitro Blue tetraxolium chloride) and purified p50 were from Promega (Madison, WI, USA). The 5′- modified oligonucleotide and its complementary strand were obtained from Keystone Laboratories (Menlo Park, CA, USA) and were synthesized in-house as described below. HPLC grade solvents were from VWR Scientific (Brisbane, CA, USA). All other chemicals were reagent grade or comparable.

2.2. HPLC columns and apparatus

Model HPXL pumps, pump heads, Rheodyne injection valves, 1.2-ml dynamic mixer, Dynamax UV-C detectors, and Dynamax Method Manager for HPLC control and data acquisition were purchased from Rainin Instrument Co. (Woburn, MA, USA). For some measurements requiring low flow-rates, the

gradient was formed in a static mixing tee obtained from Upchurch Scientific (Oak Harbor, WA, USA). Column blanks were obtained from Alltech Associates (Deerfield, IL, USA) and frits were from Upchurch Scientific.

Reversed-phase chromatography was performed on a YMC basic column (3 μm, silica-based, 150× 4.6 mm I.D.), preceded by a 23×4 mm guard column (YMC, Wilmington, NC, USA) or on a YMC C_{18} column (5 μm, 250×10 mm). An Amicon PAE column (silica-based polyethyleneimine, 10 μm, 300 Å, 100×4.6 mm), from Amicon (Beverly, MA, USA), and a Whatman Partisil 10 SAX column (250×4.6 mm) from Alltech, were used for anion-exchange separations.

2.3. Synthesis of S-triphenylmethyl-6-mercaptohexan-1-(cyanoethyldiisopropylamino) phosphite

Triphenylmethylmercaptan (25 g) was mixed with 400 ml ethanol, and a solution of 4.4 g sodium hydroxide in 25 ml of water was added. A 12.5-g amount of 6-chloro-1-hexanol was added, and the mixture was stirred for 2 h. The solution was then filtered and stripped to a tar. This material was dissolved in 300 ml methylene chloride, and the solution was washed with three 200-ml portions of water. The organic phase was dried over sodium sulfate. The resulting oil was applied to a silica column (20×4 cm) which was eluted with methylene chloride. An 8.0-g amount of pure *S*-triphenylmethylmercaptohexanol was recovered. *S*-Triphenylmethylmercaptohexanol (2.0 g) was dissolved in 100 ml of freshly distilled tetrahydrofuran and 2.0 ml diisopropylethylamine (distilled from calcium hydride) was added under argon. A 1.0-g amount of chloro-(β-cyanoethyldiisopropylamino) phosphite was added, and the solution was stirred for 2 h. The reaction was quenched by adding 20 ml of saturated aqueous sodium bicarbonate. The organic layer was dried over sodium sulfate and evaporated. The resulting material was purified over a silica column, eluting with 10% triethylamine in petroleum ether. An amount of 800 mg of *S*-triphenylmethyl-6-mercaptohexan-1-(cyanoethyldiisopropylamino) phosphite was recovered, after drying under vacuum.

2.4. Synthesis of oligonucleotides

DNA fragments were synthesized on controlled-pore glass (CPG), obtained from Biosearch Technologies (San Rafael, CA, USA), using standard β-cyanoethyl phosphoramidite methods as described by Sinha et al. [13]. An automated large-scale DNA synthesis program (Biosearch 8600) was used to synthesize 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (d22) and 5′-TTGCCTGGGAAAGTCCCCTC-AACT-3′ (d24) on 300 mg of CPG. β-Cyanoethyl phosphoramidites were obtained from Prime Synthesis (Aston, PA, USA) and tetrazole for amidite activation was obtained from Cruachem (Stirling, VA, USA); d22 was synthesized and the 5′-(4,4′-dimethoxytrityl) group was left on. The DNA was cleaved from the support and other protecting groups were removed by treatment with 5 ml of concentrated ammonium hydroxide in a closed scintillation vial for 72 h. The ammonia and water were removed in vacuo. Purification was accomplished by preparative reversed-phase HPLC. The C_{18} column was flushed with 200 ml of 25% acetonitrile in water (B), followed by 300 ml of 0.1% triethylamine adjusted to pH 7.5 with acetic acid (A). The crude DNA was dissolved in 20 ml of (A) and loaded onto the column through the (A) inlet, and was followed by 100 ml of (A). A 0–100% B gradient was run over 75 min at 12 ml/min. Fractions (10 ml) were collected and spotted on a silica TLC plate. Those giving a positive 10% $HClO_4$ reaction were pooled and lyophilized. The product was dissolved in 25 ml of 50% acetic acid and stirred for 2 h. The material was then lyophilized, and the powder was subjected to the reversed-phase procedure described above. An amount of 46 mg of purified d22 oligonucleotide was recovered which appeared homogeneous when examined by analytical reversed-phase and SAX HPLC.

The d24 oligonucleotide was functionalized on the CPG using *S*-triphenylmethyl-6-mercaptohexan-1-(cyanoethyldiisopropylamino) phosphite which was added to the last base pair of d24 with the standard large-scale automated synthesis cycle. The 5′-modified *S*-tritylmercaptohexyl oligonucleotide (tr-d24) was purified as above, without the additional acetic acid treatment, to give 50 mg of material.

2.5. Preparation and immobilization of DNA

The affinity support used for immobilization of DNA was based on 10-μm particles of crosslinked hydroxyethylmethacrylate modified with butanediol diglycidyl ether to include an epoxide functional group, as previously described [14]. Three kinds of immobilizations were studied: the immobilization of the d22 strand alone, the immobilization of the 5′-mercaptohexyl-d24 (HS-d24) strand alone, and the immobilization of double stranded DNA (ds-DNA) comprised of each of these single strands.

Immobilization of the d22 strand alone was carried out with 400 mg of support and 530 μg (74 nmoles) of the oligonucleotide in 2.7 *M* potassium phosphate, pH 7.0. At times ranging from one to six days, aliquots were withdrawn, washed with water, and the unreacted DNA was estimated from the A_{260} of the wash filtrate. In order to examine whether some d22 remained non-covalently associated with the support after the initial wash, the support was then taken up in water and transferred to a 2-ml glass vial with teflon enclosure, containing a stir bar. After heating at 85–90°C for 10 min while stirring, the product was quickly filtered. The hot water filtrate and the product were subjected to analysis as described in section 2.4.

Immobilization of HS-d24 alone was carried out for 65 h with 200 mg of support and 800 μg (103 nmoles) of oligonucleotide in 2.7 *M* potassium phosphate, pH 7.0. After the 65-h period a sample of the supernatant from the reaction was injected onto the reversed-phase column, indicating that >95% of the DNA was associated with the support. The product was washed with water and acetone, air-dried, and subjected to analysis as described in section 2.4 (note: in this reaction the wash filtrate was not examined for unreacted DNA). The HS-d24 required for the reaction was generated immediately beforehand from silver nitrate treatment of *S*-tritylmercaptohexyl-d24 (tr-d24) as described in Ref. [15]. Briefly, five mole-equivalents of silver nitrate were added to tr-d24 in water. After 30 min this was followed by the addition of a two-fold excess of dithiothreitol to precipitate the silver. After 30 min the mixture was centrifuged and the HS-d24 was recovered in the supernatant.

For immobilizations of ds-DNA, the d22 and HS-d24 strands were first annealed by combining equal amounts of HS-d24 and d22 in a final concentration of 1.0 *M* sodium chloride. The solution was heated at 85°C for 5 min followed by 45°C for 45 min and cooling to room temperature. Hybridization of the strands was corroborated by the disappearance of peaks in anion-exchange and reversed-phase separations corresponding to the individual strands and the appearance of new peaks thought to be the duplex.

The immobilization of ds-DNA with time was examined for mixtures of 10 mg of support and 46 μg (about 6.0 nmole) of ds-DNA. Reactions were allowed to proceed from one to three days in 2.7 *M* potassium phosphate, pH 7.0. The products were filtered and washed with 3 ml of 1.0 *M* NaCl. Unreacted DNA was estimated from injection of the wash filtrate on the reversed-phase column. The product was taken up in water and subjected to the hot water treatment as described above for d22. The DNA released was estimated from reversed-phase analysis of the hot water filtrate (melt).

In another series of measurements, the effect of salt concentration on the immobilization efficiency of ds-DNA was estimated. Reactions were carried out with 120 mg of activated support and 92 μg of ds-DNA, in potassium phosphate concentrations at 50 m*M* and between 1.0 and 3.0 *M*, at either pH 7.0 or 8.0. After 120 h the mixtures were filtered and washed with 10 ml of 1.0 *M* NaCl. Unreacted DNA was estimated from the A_{260} of the wash filtrate.

All immobilizations were performed at room temperature, in a volume of 1–2 ml. DNA solutions were prepared by dissolving DNA in water at about 5 μg/μl and adjusting the DNA and salt concentrations to reaction conditions by the addition of appropriate volumes of 3.0 *M* potassium phosphate, 4.0 *M* sodium chloride, and water. Concentrations of ss-DNA were based on the assumption that an absorbance of 1.0 AU at A_{260} corresponded to a concentration of 33 μg/μl.

2.6. Acid hydrolysis and nuclease P1 digestion of immobilized DNA

The product from the d22 immobilization, following the hot water treatment, was subjected to acid hydrolysis. The hydrolysate was then digested with

nuclease P1, and the products were examined by reversed-phase chromatography. Acid hydrolysis results in cleavage of relatively labile purine–glycosidic linkages and would thus result in the release of those purine bases which have not reacted with the support. In addition to cleaving the purine bases, limited hydrolysis of phosphodiester bonds also occurs. Since some of these fragments overlap with guanine and adenine peaks in the reversed-phase separation, a subsequent nuclease P1 digestion was carried out on the acid hydrolysate in an attempt to reduce the apurinic acid fragments to mononucleotides, which are easily distinguished from the purines in the reversed-phase separation.

Acid hydrolysis of the d22 support was carried out at 85–90°C in 0.1 *M* sulfuric acid for two hours while stirring, using 2-ml glass vials with teflon enclosures. The mixture was then filtered and the filtrate (hydrolysate) neutralized with sodium hydroxide. Nuclease P1 digestions of the hydrolysate, containing 2.5 μg/ml of enzyme (approximately 300 units activity per mg), were performed at room temperature in 50 m*M* sodium acetate (pH 5.4) and 0.5 m*M* zinc sulfate. Digestions were generally complete after 30 min, but were occasionally allowed to proceed overnight. The enzymatic digests were injected directly onto the reversed-phase column equipped with a guard column. Quantitation of purine base and pyrimidine mononucleotide concentrations from peak areas was based on response factors determined from independent injections of the appropriate standards.

The wash filtrates and hot water filtrates from the d22 immobilizations were also subjected to nuclease P1 digestion and subsequent reversed-phase analysis. The wash filtrate was found to resist digestion, possibly from the presence of the salt initially present in the reaction mixture. Reversed-phase analysis of the nuclease P1 digestion from the hot water filtrate showed the four mononucleotides and adenosine in proportions consistent with intact d22.

The product from the HS-d24 immobilization was subjected to acid hydrolysis–nuclease P1 digestion and subsequent reversed-phase analysis as described for the d22 product.

The nucleotide compositions of the d22 and tr-d24 were confirmed from reversed-phase analyses of the mononucleotides generated from digestion with nuclease P1. The empirical compositions obtained were within about 5% of the values predicted from the theoretical compositions; similar results were obtained for DNA synthesized in-house and that obtained externally. Representative chromatograms are shown in Fig. 1a for the d22 digest and Fig. 1b for the tr-d24 digest.

In order to test the validity of the acid digestion–nuclease P1 procedure, known amounts of d22 and tr-d24 were treated as described above. Recovery of purine bases and pyrimidine mononucleotides were then examined by reversed-phase analysis. As shown in Fig. 2a, the d22 acid hydrolysate exhibited three major bands along with a few smaller peaks. Two of the major peaks were identified as guanine and adenine; the other major peak is thought to be a form of apurinic acid which had undergone limited hydrolysis to produce a few minor degradation fragments, appearing as the smaller peaks. The nuclease P1 digestion, seen in Fig. 2b revealed the disappearance of several minor peaks and the appearance of two new peaks which were found to co-elute with 2′-deoxycytidine-5′-monophosphate (dCMP) and thymidine-5′-monophosphate (TMP). However, their recoveries were considerably less than that in the starting material. The third major peak observed in the acid hydrolysate was also present. This species apparently resists nuclease P1 degradation and probably accounts for the missing dCMP and TMP. Quantitative recovery was obtained for the purine bases.

Comparable results were obtained for acid hydrolysis and subsequent nuclease P1 digestion of tr-d24, as seen in Fig. 2c and 2d, respectively. The tr-d24 hydrolysate showed the guanine and adenine peaks and a large number of smaller peaks. The nuclease P1 digestion resulted in quantitative recovery of the purines but only partial recovery of the pyrimidine mononucleotides, although recovery of the latter was somewhat higher than that observed for d22. Apparently, the apurinic acid fragments produced from tr-d24 acid hydrolysis undergo nuclease P1 digestion more readily than those from the d22 hydrolysis. It is interesting to note that the third major peak, which was observed in the d22 acid hydrolysate (Fig. 2a), was absent in the tr-d24 hydrolysate (Fig. 2c). In separate measurements (data not shown), when acid hydrolysis of tr-d24 was

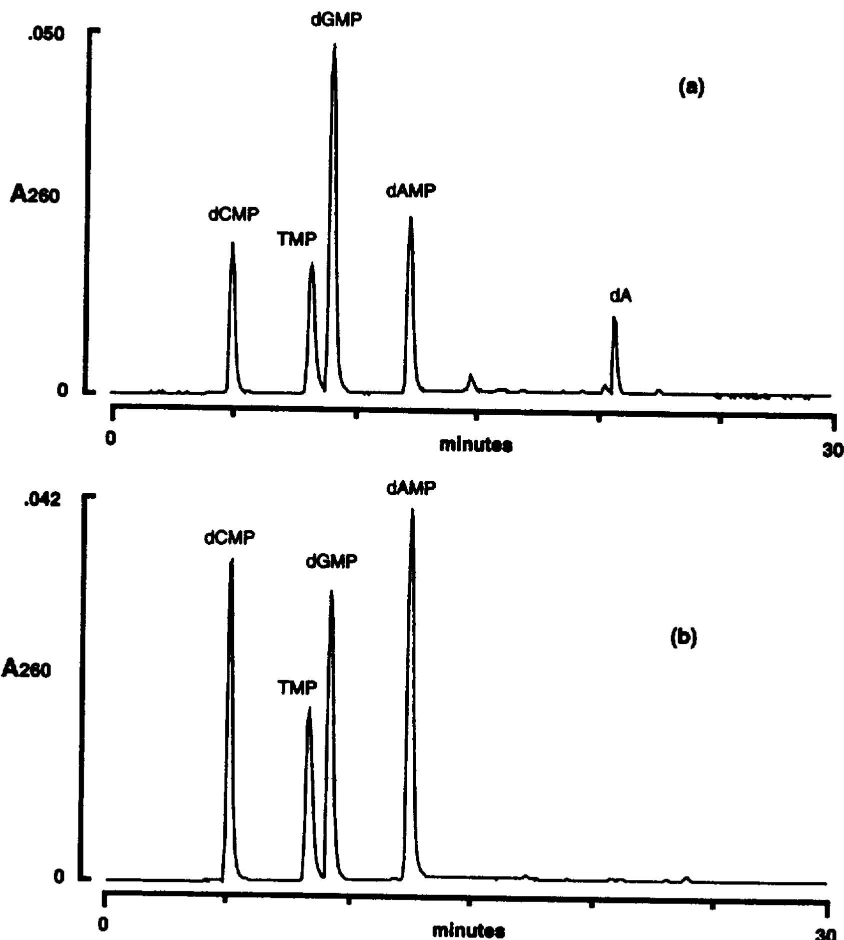

Fig. 1. Reversed-phase analysis of nuclease P1 digests from (a) d22 and (b) tr-d24 oligonucleotides. From the analyses were calculated the oligonucleotide base compositions which were found to compare closely with the theoretical compositions for both oligonucleotides. (a) The empirical composition as calculated from the analysis of the d22 digest was $C_{5.05}T_{4.88}G_{8.00}A_{3.08+0.90}$ as compared with a theoretical composition for d22 of $C_5T_5G_8A_{3+1}$. The values represent mononucleotides except "A" which is the sum of the monophosphate and adenosine which is released from the 3′ terminus. (b) The empirical composition of tr-d24 was calculated as $C_8T_{5.02}G_{5.00}A_{5.25}$ as compared with a theoretical composition of $C_8T_5G_5A_6$. The 5′ terminal dAMP remains attached to the tritylmercaptohexyl group after the digestion and does not elute under these conditions. Column: YMC basic with guard, 150×4.6 mm; pump A: 10 m*M* sodium phosphate, pH 6.9; pump B: methanol; method: 0–30 min, 0–100% B; flow: 0.5 ml/min; detection: 260 nm. Peaks: dCMP=2′-deoxycytidine-5′-monophosphate, TMP=thymidine-5′-monophosphate, dGMP=2′-deoxyguanosine-5′-monophosphate, dAMP=2′-deoxyadenosine-5′-monophosphate, dA=2′-deoxyadenosine.

carried out for one instead of two hours, this peak was present.

2.7. Affinity chromatography and dot-blot analysis

Double stranded DNA affinity supports were slurry packed into either 30×2.1 mm or 50×4.6 mm stainless steel columns fitted with 2-μm titanium frits. Sodium chloride (1.0 *M*) was used as drive and slurry solvent. Packing was carried out with an HPLC pump at 2000–3000 p.s.i. (13.8–20.7 MPa). Nuclear extracts or purified p50 protein were loaded onto the column in a buffer system consisting of 10% glycerol, 0.01% Nonidet P40, 100 m*M* EDTA,

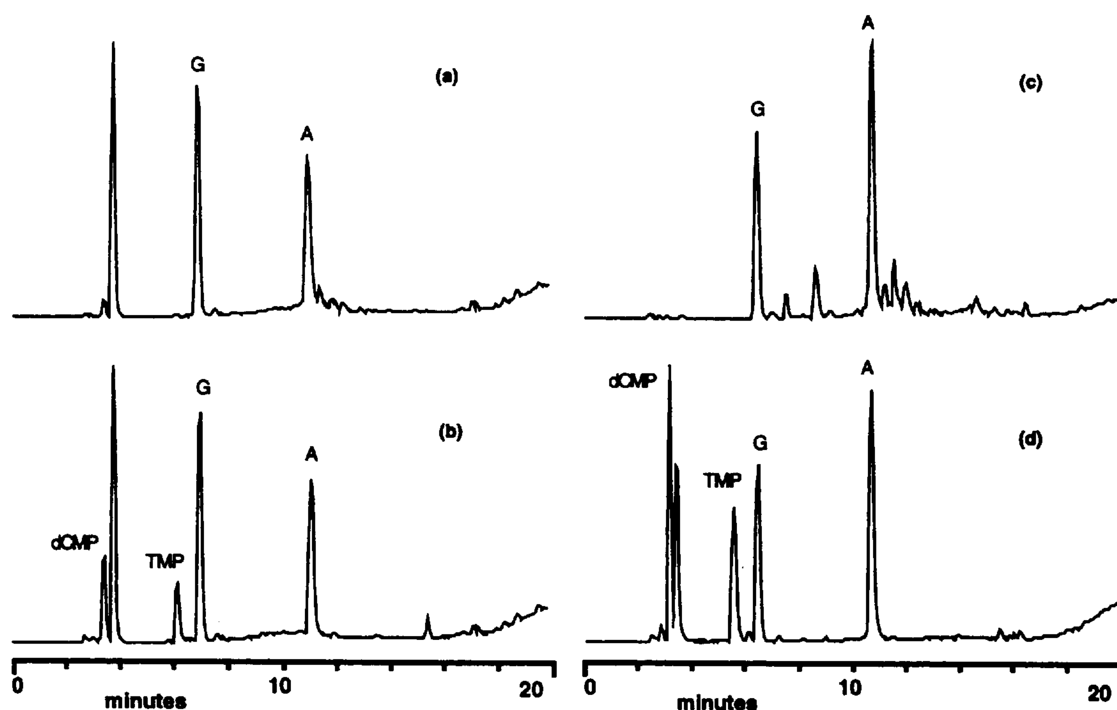

Fig. 2. Reversed-phase separation of the acid hydrolysate and of the subsequent nuclease P1 digestion of d22 and tr-d24. Complete recovery of purines was obtained but only partial recovery of pyrimidines. See text for details. (a) and (b) Acid hydrolysate and its nuclease P1 digestion, respectively, for d22. (c) and (d) Acid hydrolysate and its nuclease P1 digestion, respectively, for tr-d24. Column: YMC basic with guard, 150×4.6 mm; pump A: 10 m*M* sodium phosphate, pH 6.5; pump B: methanol; method: 0–7.6 min, 0–20% B; 7.6–16 min, 20–100% B; 16–20 min, 100% B; flow: 0.75 ml/min; detection: 260 nm. Peaks: dCMP and TMP as in Fig. 1, G=guanine, A=adenine. The unlabeled major peaks eluting early in (a), (b) and (d) are thought to correspond to a form of apurinic acid.

1 m*M* dithiothreitol, 150 m*M* NaCl, and 25 m*M* HEPES at pH 7.5. After loading for several minutes the flow-rate was increased (typically from 0.1 to 1.0 ml/min), and the column was washed until the absorbance leveled off near baseline (50–150 column volumes). The column was then eluted in a linear gradient from loading buffer to 1000 m*M* sodium chloride in loading buffer. Fractions were collected over the entire run and were tested for the presence of the p50 subunit using a "dot-blot" format. In this procedure a sample of the fraction was applied to a nitrocellulose membrane which was then treated with non-fat dry milk to block non-specific adsorption sites. The membrane was then treated with rabbit anti-human p50 polyclonal antibody. After a Tween wash, alkaline phosphatase conjugated goat anti-rabbit antibody was applied to the membrane. After another Tween wash the spots were developed using the BCIP/NBT substrate system.

3. Results

3.1. Immobilization of d22

Reaction of the d22 strand with the HEMA-epoxide support was carried out in 2.7 *M* potassium phosphate, and the progress of the reaction was examined at 20, 44, 68 and 136 h. The amount of unreacted d22, determined from the A_{260} and from reversed-phase injections of the wash filtrate and hot water filtrate (see Experimental), declined from 43% of the initial amount at 20 h to less than 15% unreacted at 136 h. The yield of 85% observed at 136 h corresponds to a coverage of 0.16 μmoles per

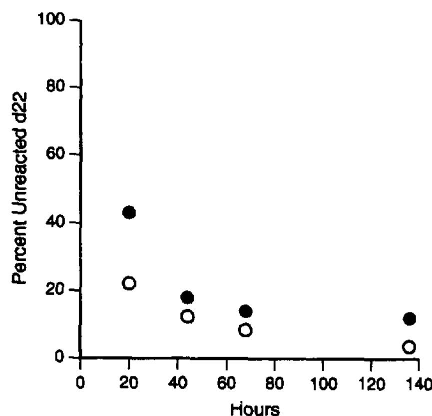

Fig. 3. Unreacted d22 oligonucleotide following the reaction with the epoxide affinity support. After washing the product with water, a significant portion of d22 remains non-covalently associated with the support and is only released after treating with hot water. See text for details. (●) Total d22 recovered after water wash and subsequent hot water treatment; (○) d22 recovered after treatment with hot water. The difference in the two values represents d22 appearing in the wash preceding the hot water treatment.

gram. As seen in Fig. 3, a significant portion of the unbound material remained associated with the support after the initial filtration and water wash step, and was released only after the hot water treatment. The nucleotide composition of the hot water filtrate, determined from reversed-phase analysis of a nuclease P1 digestion, reflected the composition of intact d22. This suggests a non-covalent association, possibly due to intermolecular association with covalently bound d22.

After the treatment with hot water the support was subjected to acid hydrolysis, and the hydrolysate was digested with nuclease P1. In contrast with earlier measurements for d22, in which five major peaks were present in the reversed-phase separation, only four were observed in these digests. The chromatograms for the 20-h and 136-h digests are shown in Fig. 4. Reversed-phase analysis indicated that only about 60% of the bound purines were recovered in the hydrolysate, assuming that the amount of d22 reacting with the support is given by the difference between the starting material and that recovered in the water washes. About 40% of bound TMP was recovered. Recovery of dCMP ranged from nearly 70% after 20 h down to 32% at 136 h. Recoveries for the four time points are shown in Fig. 5.

3.2. Immobilization of HS-d24

After washing and filtration, the product from the HS-d24 immobilization was subjected to acid hydrolysis and the hydrolysate was in turn digested with nuclease P1. Reversed-phase analysis of the enzymatic digest showed the five peak pattern observed for tr-d24 (Fig. 2d). The recoveries of guanine, adenine, dCMP and TMP were about 75% each for guanine and adenine, 45% for dCMP, and 50% for TMP, based on the initial amount of HS-d24 in the reaction. This indicates that no more than 25% of the purines reacted with the epoxide; the actual extent of purine reaction could have been less, depending on the amount of unreacted DNA in the wash (the wash from this reaction was not examined for DNA). Although >95% of starting material was found to be associated with the support after 65 h reaction, this does not necessarily indicate that the associated DNA was covalently bound; in the presence of high salt concentration, an induced hydrophobic association with the support would be expected (see Discussion).

An attempt to form the double stranded duplex by combining immobilized HS-d24 with excess d22 in water under annealing conditions failed: examination of the supernatant on reversed-phase before and after annealing showed no difference in d22 concentration. That the strands were capable of forming the duplex was supported by annealing equimolar concentrations of the strands in 1.0 *M* NaCl and examining the mixture by reversed-phase and anion-exchange chromatography. As seen in Fig. 6a, the reversed-phase separation showed a third peak which was distinct from either HS-d24 or d22 and eluted between them. Only about 50% of the peak area appears as the third species, which probably represents ds-DNA. The distribution of the strands among the three forms could be influenced by reversed-phase conditions and may not represent their arrangement prior to injection. Similar results were obtained for a mixture of tr-d24 and d22, seen in Fig. 6b. Injections of the annealed HS-d24/d22 mixture on the anion-exchange column (Fig. 7c) showed a broad, tailing band eluting at a time different than that for either strand alone (Fig. 7a and 7b). The peaks corresponding to the single strands are absent for the annealed mixture. The elution of the duplex

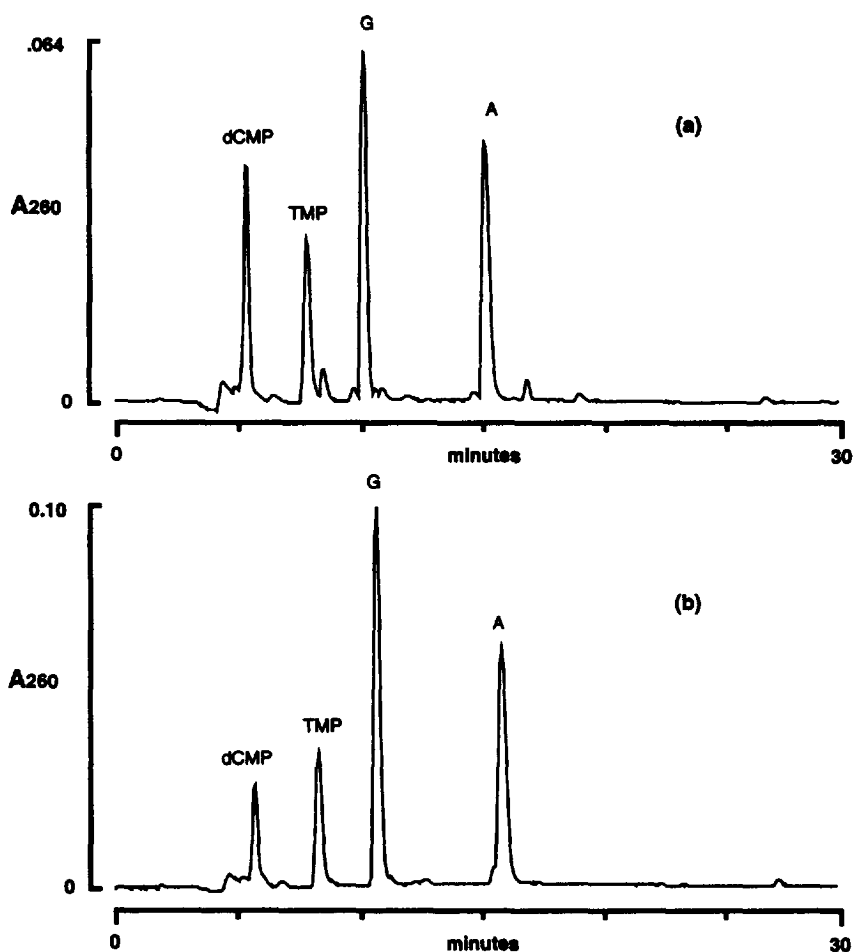

Fig. 4. Reversed-phase separation of nuclease P1 treated acid hydrolysate of immobilized d22 oligonucleotide. The product from the reaction between d22 oligonucleotide and the epoxide support was washed with water and treated with hot water to remove non-covalently associated d22, as described in the text. The immobilized d22 product was then subjected to acid hydrolysis and the hydrolysate to enzymatic digestion. (a) Digestion after 20 h reaction; (b) Digestion after 136 h. Conditions for the reversed-phase separations were as described in Fig. 1.

at the earlier time indicates that it actually experiences a weaker interaction with the stationary phase than either of the single strands, notwithstanding a much larger phosphate content in the duplex.

3.3. Immobilization of double stranded DNA

A plot of unreacted ds-DNA against potassium phosphate concentration is shown in Fig. 8 for reactions carried out at pH 7; similar results were obtained at pH 8. The extent of immobilization of ds-DNA was sensitive to the concentration of potassium phosphate in the reaction. At salt concentrations approaching 3 *M*, only about 5% of the initial DNA was recovered in the wash filtrate, as estimated from the A_{260}. As the salt concentration dropped there was a substantial increase in the amount of DNA in the filtrate.

Immobilization of ds-DNA, carried out in 2.7 *M* potassium phosphate, was examined at 22, 44, and 70 h. As shown in Table 1, reversed-phase analysis of the wash filtrate showed progressively lower amounts of unreacted DNA in the wash filtrate, and by 70 h about 90% (0.30 μmoles/g) was associated with the support. Reversed-phase analysis of the

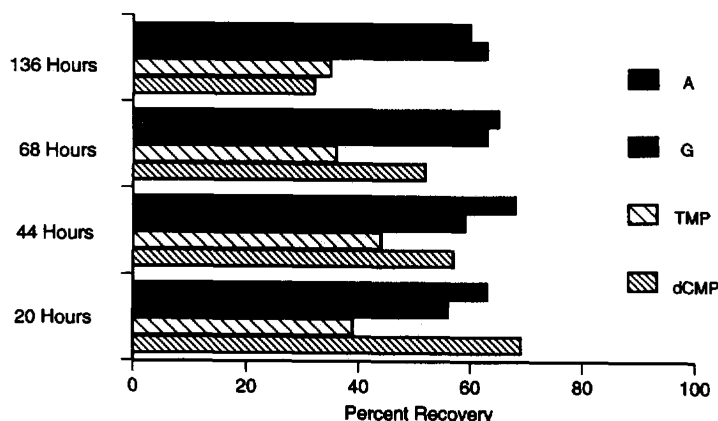

Fig. 5. Percent recovery of pyrimidine nucleotides and purine bases from immobilized d22. The recovery is given as a percent of the covalently bound material, i.e. the amount calculated from the difference between the starting material and that recovered in the water wash/hot water treatment as described in Fig. 4. The recovery was determined from reversed-phase analysis following the acid hydrolysis–nuclease P1 digestion procedure described in the text. Reversed-phase conditions as described for Fig. 1.

wash filtrate at 22 h showed the three-peak pattern of Fig. 2a corresponding to d22, ds-DNA, and HS-d24 in an approximate peak area ratio of 2:2:1. By 44 h the ds-DNA and HS-d24 peaks were absent.

Reversed-phase injections of the melt filtrates showed that at 22 h ds-DNA and HS-d24 accounted for about 15% and 10% of peak areas, respectively; in subsequent reactions these species were absent indicating that the DNA released in the melt corresponded to the d22 strand. At 44 and 70 h, the recovery of d22 in the melt represented 85–90% of the d22 which was associated with the support after the wash step.

The product from the 70-h reaction was subjected to the annealing procedure, in 1.0 *M* NaCl, after carrying out the melt procedure. The DNA released in the melt failed to recombine with the support.

3.4. Injections of p50 and nuclear extract onto the NF-κB affinity column

Dot-blot results are shown in Fig. 9a and 9b, respectively, for injections of purified p50 protein and nuclear extract from phorbol ester-stimulated HeLa cells onto the ds-DNA affinity column. p50 was detected in both unretained and retained fractions.

4. Discussion

In this study immobilizations of single stranded and double stranded DNA onto a HEMA-based, epoxide-activated HPLC support were obtained in high yields when reactions were performed in the presence of high potassium phosphate concentrations. For double stranded DNA, the extent of immobilization began to increase at salt concentrations above 1.0 *M*; at 2.5–3.0 *M*, nearly total immobilization was obtained. We have obtained similar results for high-salt immobilizations of proteins on a silica-based, epoxide-activated affinity support [12] and for immobilizations of short polypeptides on the polymeric support used in this study (unpublished results). The extent of protein immobilization was found to increase with salt concentration, as was observed for DNA immobilizations in the present study. This phenomenon can be attributed to increased reactivity resulting from salt dependent partition of the solute along the surface of the support, analogous to the salt-dependent retention which occurs in hydrophobic interaction chromatography. The concentration threshold at which protein immobilization was found to increase varied according to the individual protein [12]: some proteins began to show increases at concentrations below 0.5 *M* while others required greater that 1.0 *M* to show

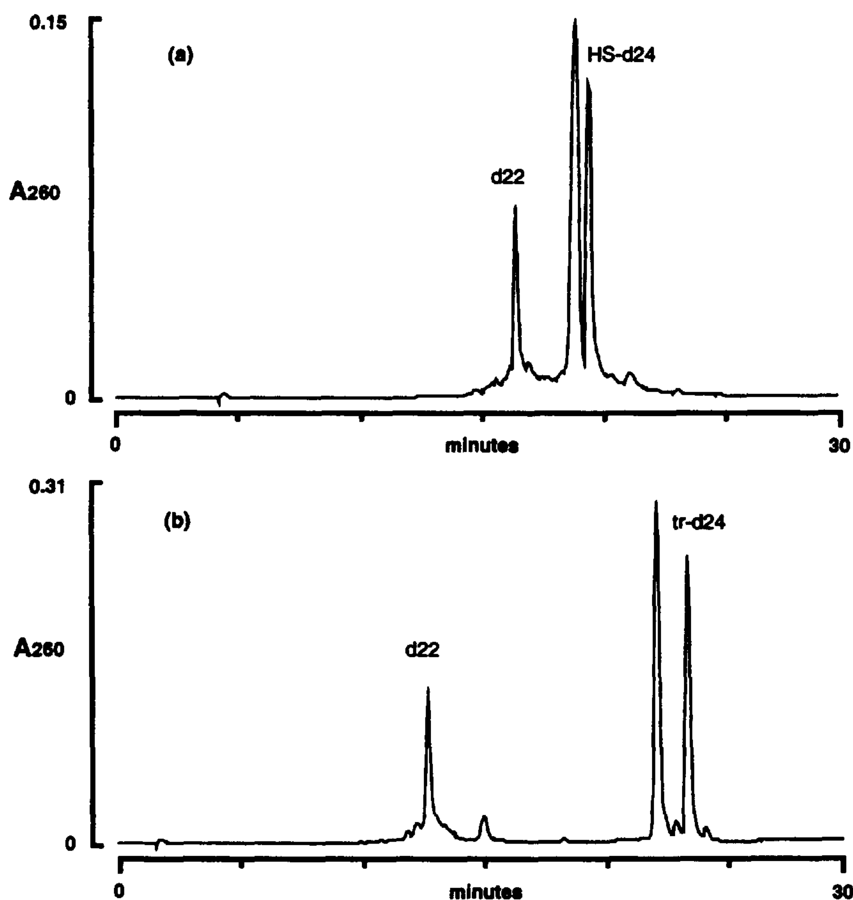

Fig. 6. Reversed-phase analysis of annealed (a) d22, HS-d24 mixture, and (b) d22, tr-d24 mixture. The middle peak in each case is thought to be double stranded DNA. Conditions as in Fig. 1 except in (b) the gradient is 0–100% B over 30 min instead of 0–50% B over 30 min as in (a).

this effect. For salt-induced immobilizations of DNA, similar differences in concentration thresholds might be encountered according to variations in DNA composition and size.

Initial attempts to immobilize the thiol-modified strand (HS-d24) in 0.50 *M* sodium carbonate, pH 9.1, resulted in low levels of coverage. Similar results were reported by Blanks and McLaughlin for reactions of a thiopropyl dodecamer with epoxy- and tresyl activated Sepharoses [8] carried out at pH 10. In that study, 15–20% of the DNA was immobilized. The coverage was estimated at 0.3 μmoles/ml, beginning with a support possessing 15–20 μmoles/ml of epoxide. Recovery of purines from the HS-d24 modified support in this study indicated that at least 75% of the initial material reacted with the support, which would represent a coverage of 0.50 μmoles/g (about 0.30 μmoles/ml). It must be noted that some of the recovered purine could have arisen from non-covalently associated ligand, as was observed in the reactions with the d22 strand. However, this amount is probably small since only about 9% of the d22 was found associated in this manner after a comparable reaction time. The yield of about 85% obtained in the d22 immobilization represents a coverage of 0.16 μmoles/g of support.

The mode of attachment of DNA to the support represents an important question. Salt-induced as-

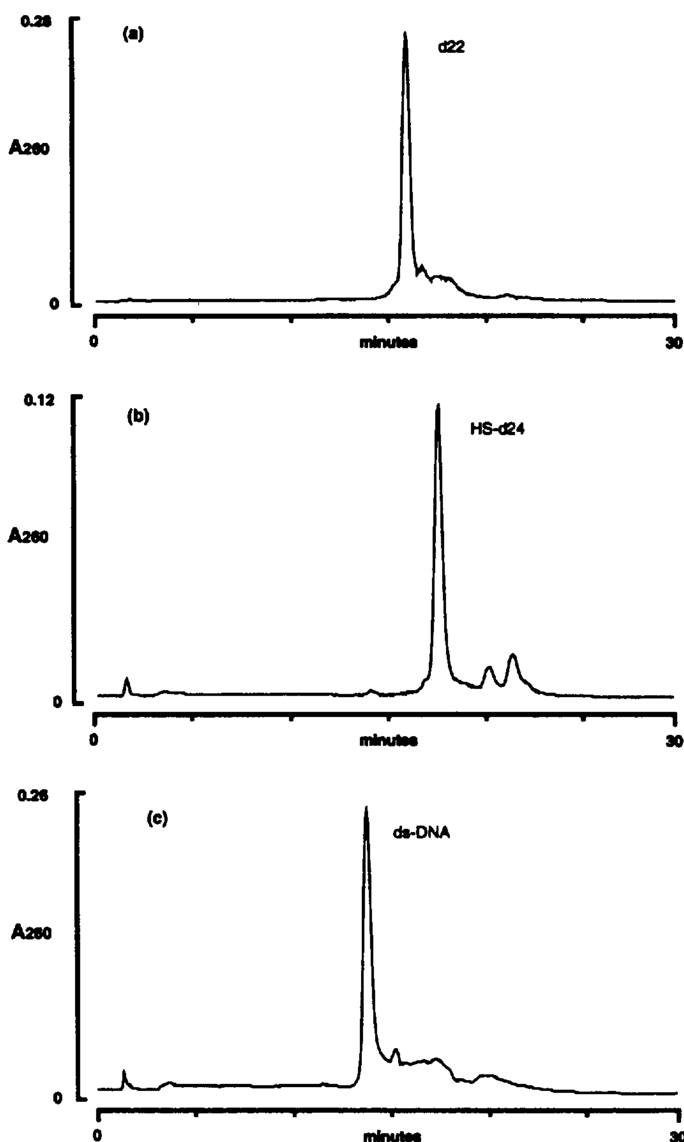

Fig. 7. Anion-exchange of (a) d22 oligonucleotide, (b) HS-d24 oligonucleotide, and (c) the annealed mixture. The absence of the peaks corresponding to the single stranded species in (c) suggests the formation of the duplex. Column: Amicon PAE, 100×4.6 mm; pump A: 20% acetonitrile in 10 m*M* sodium phosphate, pH 7.0; pump B: 1.6 *M* sodium chloride in A. Method: 0–100% B over 30 min; flow: 1.00 ml/min; detection: 260 nm.

sociation of the DNA with the support would be expected to bring the nucleotide bases into reactive proximity to the epoxides resulting in base attachment, an outcome supported by the d22 results. Hydrolysis of the d22 product returned only about 60% of the immobilized purines. The fact that good recoveries of purines were obtained from hydrolyzed d22 (in solution) suggests that, during the immobilization, 40% reacted with the support, assuming that no reaction occurred during the hydrolysis step. Less can be concluded about the extent of pyrimidine attachment. Under the relatively mild conditions used in these procedures, sequences of consecutive pyrimidyl structure resist cleavage [16,17], a fact

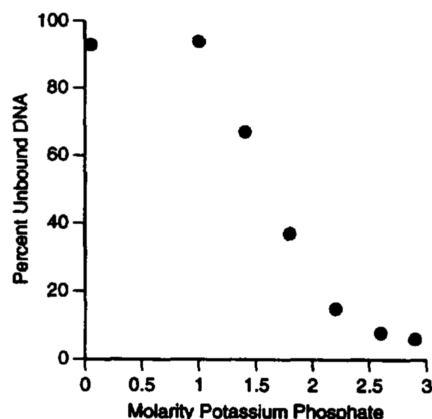

Fig. 8. Percent unreacted, double stranded DNA plotted against the concentration of potassium phosphate in the reaction. The results indicate that the amount of d22 which remains associated with the support increases with salt concentration, exceeding 90% of starting material at high salt concentration. Annealed d22, HS-d24 was reacted with the epoxide support for 65 h in various concentrations of potassium phosphate. After washing the product, the unreacted DNA was determined from the A_{260} of the product wash.

borne out from the hydrolysis of d22 and tr-d24. As a result, attachment at any point along such a sequence would tend to immobilize contiguous pyrimidines, thereby precluding information concerning the extent of attachment. Exhaustive hydrolysis of all phosphodiester bonds could provide more information on this point, but such a course was not pursued in this study.

End-point attachment through the sulhydryl is obviously desirable in order to avoid perturbation of the protein binding site. Preferential attachment at

Table 1
Immobilization of double stranded DNA

Time (h)	22	44	70
Wash filtrate (μg)	11.6	5.2	3.2
Melt (μg)	14.9	15.8	17.1

Double stranded DNA, prepared from annealing 23 μg each of the complementary oligonucleotides d22 and HS-d24, was reacted with the epoxide support in 2.7 *M* potassium phosphate for various times as described in the text. The DNA recovered in the wash filtrate represents unreacted DNA. The DNA released in the hot water treatment (Melt – see Experimental) represents predominantly d22, thought to be hybridized to the immobilized HS-d24 strand. The values given below were determined from direct injection onto the reversed-phase column using the conditions decribed in Fig. 1.

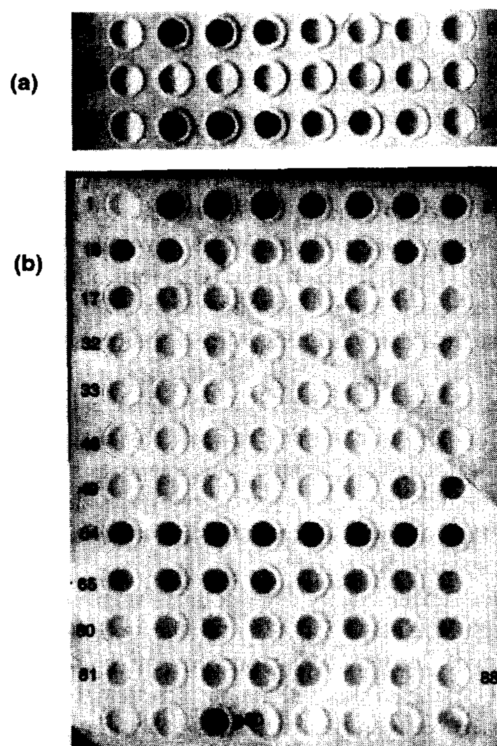

Fig. 9. Dot-blot analysis for p50 in the eluate obtained from the injection of (a) p50 purified protein or (b) nuclear extract from phorbol stimulated HeLa cells onto the NF-κB affinity column. Selected fractions representing the flow-through, wash, and salt eluate were tested for p50 using a dot-blot format as described in the text. The dark spots indicate a positive test for p50. For both (a) and (b) the initial spots correspond to unretained p50. These are followed by fractions collected during the column wash which did not test positive for p50. The final series of spots correspond to p50 eluted from the column in a salt gradient. A steep salt gradient was used for the (a) elution resulting in concentrated p50 fractions. A shallow gradient was used for (b). Purified p50 standard is spotted in the last row of 9b.

the 5′- terminus might be improved by increasing the hydrophobicity of the spacer arm adjacent to the sulfhydryl. In that case association with the support could be shifted toward the terminus, possibly at concentrations of salt low enough to minimize contact along the DNA. Alternatively, immobilization of concatenated DNA could result in attachment at a limited number of the available copies, leaving others unobstructed for protein associations. Particles with very wide pores (1000 Å or larger) would be recommended in order to accommodate the im-

mobilization of longer ligands and their binding partners. Perfusion-type particles might also be worth examining for these purposes.

Affinity supports possessing the double stranded DNA demonstrated binding for p50, a subunit of the transcription factor NF-κB. The p50 subunit has been shown to bind to DNA recognition sites, both as a homodimer and in association with the rel A subunit [18]. p50-positive results were obtained for the retained fractions from injections of purified p50 and nuclear extracts derived from phorbol stimulated HeLa and Jurkat T cells. Injections of p50 and nuclear extracts also showed a positive reaction for p50 in the unretained fractions. This suggests a partially denatured sample which is consistent with the product description provided by the supplier of the p50 protein in which the preparation is stated to be only partially active.

The attachment of the double stranded ligand through the DNA bases, to the extent that it occurred, apparently did not prevent binding. The results indicate that although substantial reaction occurred between the epoxide and DNA bases in the immobilization of d22 (60% recovery of purines), most of the d22 associated with the support in the double stranded DNA immobilizations was recovered in the melt (85–90%). This suggests that the bases are less reactive when the d22 strand is hybridized to its complementary strand; intermolecular organization of the two strands restricts the way in which the bases are presented at the surface of the support. In the case of immobilized single stranded HS-d24, attachment through the bases could interfere with d22 hybridization which would explain the failure of immobilized HS-d24 to take up the d22 strand. The reason for the failure of d22 to reanneal after applying the melt procedure to immobilized double stranded DNA is not clear.

In conclusion, the presence of high concentrations of salt in the reaction medium leads to high-yield immobilizations of DNA, probably resulting from high localized concentrations of the reactants at the surface of the support. This is a general phenomenon resulting from salt-induced surface adsorption and should be applicable to other kinds of chromatographic supports and to a variety of activation chemistries.

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