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CONSTRUCTION OF BRANCHED DNA (bDNA) MOLECULES BY CHEMICAL LIGATION

Nina Dolinnaya #*, Sergei Gryaznov ‡, David Ahle ¥, Chu-An Chang ¥,
Zoe A. Shabarova #, Mickey S. Urdea ¥, and Thomas Horn ¥*

¥ Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608, USA
‡ Department of Chemistry, Northwestern University, Evanston, IL 60208, USA. Present address: Lynx Pharmaceuticals, Foster City, CA 94404, USA.
# Chemical Department, Moscow State University, Moscow 119899, Russia

ABSTRACT. Chemical ligation has been investigated in the assembly of branched DNA (bDNA) molecules. Water soluble coupling reagents were used to ligate single-stranded linear oligomers to bDNA molecules containing 5 and 15 branches. The ligations were particularly efficient when one of the oligomers was partially double-stranded constructed by branching to create a terminal ligation linker (AutoLink). Branched DNA molecules containing more than 1000 nucleotides were assembled.

INTRODUCTION

We have developed signal amplification as a strategy for achieving greater sensitivity in nucleic acids hybridization assays [1]. The technique is based upon the specific hybridization of branched DNA (bDNA) and enzyme labeled probes to nucleic acid targets such as hepatitis B DNA, hepatitis C RNA, and human immunodeficiency virus RNA. The bDNA amplifiers contained a single, unique oligonucleotide sequence (the primary sequence) covalently linked to several copies of a different sequence (the secondary sequence). The primary sequence was designed to hybridize specifically with the target nucleic acid and the secondary sequences to hybridize with enzyme-labeled probes.

The bDNA molecules were assembled using a combination of solid phase chemical synthesis and enzymatic ligation methods [2]. We have developed methods for the chemical synthesis of bDNA oligomers on solid supports using a branching monomer (BM) [3]. The BM is a phosphoramidite reagent derived from N^4-(6-hydroxyhexyl)-5-methyl-2'-deoxycytosine where the hydroxyl function is protected as the levulinate. The chemical synthesis produced a comb-shaped bDNA molecule (bDNA comb) which had a unique primary sequence covalently attached through BMs to a number of identical secondary sequences, each containing 6 nucleotide residues. The bDNA comb was further elaborated into a bDNA amplifier by enzymatic ligation in which long, linear oligonucleotides, in the presence of a complementary linker, were added to all the secondary sequences of the bDNA comb [2].

We were interested in investigating chemical ligation as a way of assembling bDNA amplifiers. Chemical ligation has been developed as a complement to enzymatic ligation [4], and the technique has added advantages. It could be used to obtain DNA duplexes containing both natural and modified internucleotidic linkages [4,5]. The chemical coupling technique has been applied in the construction of modified oligomers [4], circular DNA molecules [6,7], and in the assembly of a biologically active gene containing 183 base-pairs from chemically synthesized oligonucleotides [8].

We have found that chemical ligation may be used in the construction of bDNA molecules. In this paper we report results of the assembly of several bDNA amplifiers containing either natural phosphodiester or modified (pyrophosphate and phosphoramidate) internucleotidic linkages at the ligation sites.

MATERIALS AND METHODS

General.

1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDC) and a 5 M solution of cyanogen bromide (BrCN)in anhydrous acetonitrile were obtained from Merck.

Analysis of DNA oligomers were performed using polyacrylamide gel electrophoresis (PAGE) and UV shadowing and high performance capillary electrophoresis (HPCE). Quantitation of UV_{254} detected bands by HPCE analysis was used to estimate coupling yields.

Oligodeoxyribonucleotide Synthesis.

Oligodeoxynucleotides were synthesized by standard solid-phase chemistry using 2-cyanoethyl phosphoramidite monomers [9]. The phosphorylating reagent 2-[[2-[(4,4'-dimethoxytrityl)oxy]ethyl] sulfonyl

ethyl- 2-cyanoethyl-N,N-diisopropylphosphoramidite [10] was used to synthesize 3'- and 5'- phosphorylated oligomers. For synthesis of 3'-phosphates, the phosphorylation reagent was coupled to a T-support during the first synthesis cycle. Upon completion of the synthesis, standard ammonium hydroxide deprotection cleaved the linker directly generating the free 3'-phosphate (oligomer-3'-p) of the desired oligonucleotide. For synthesis of 5'-phosphates (5'-p-oligomer) the phosphorylating reagent was added during the last synthesis cycle. The monomethoxytrityl derivative of 5'-amino-5'-deoxy-thymidine [11] was converted to the 2-cyanoethyl phosphoramidite and used in the normal fashion [9].

A three-oligomer system consisted of a 24-mer terminating in GCG-TAG-3'-p (1) which was ligated to a 33-mer terminating in 5'-XGA-CTG, where X = 5'-HO-T (2), 5'-NH_2-T (3), or 5'-p-T (4), in the presence of the linker molecule 5'-CAG-TCA-CTA-CGC-3' (5).

Branched oligonucleotides were synthesized essentially as described [3]. The assembly started with the synthesis of the primary sequence: 5'-(TT-BM)$_n$-TTT-TTT-TTT-GAC-ACG-GGT-CCT-ATG-CCT-3', where T indicates the thymidine-CPG solid-support, and n = 4 for combs 7, 8, and 9, and n = 14 for comb 10. The structure of the BM nucleoside in its phosphoramidite form is shown in Fig. 1. The individual solid-supported fragments were next treated with 0.5M hydrazine hydrate in pyridine/acetic acid (4:1 v/v) for 90 minutes to remove the levulinate protection group on the exocyclic hydroxyl group of the BM nucleotides [3]. Chemical DNA syntheses were then continued off the terminal 5'-hydroxyl and the liberated hydroxyl groups to assemble multiple, identical copies of the secondary sequence: 5'-XGA-CTG-p-3'. The outlined synthesis strategy was used to assemble bDNA combs 7, 8, and 9 with five secondary sequences (5x bDNA; n = 4), where X = 5'-NH_2-T (7), 5'-p-T (8), and 5'-HO-T (9), and the bDNA comb 10 containing 15 secondary sequences (15x bDNA; n = 14), where X = 5'-NH_2-T (10). The chemical synthesis of a bDNA comb containing n secondary sequences is outlined in Fig. 3.

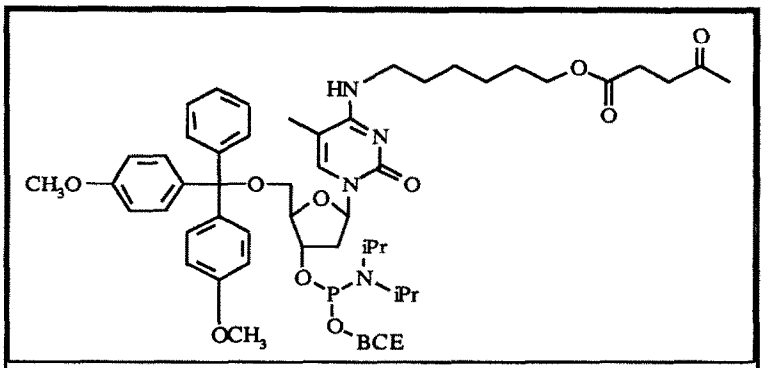

Fig. 1. *The structure of the BM phosphoramidite.*

Novel singly-branched oligonucleotides, AutoLink molecules, were synthesized where the primary sequences were designed so they could form an internal hairpin with a 6 nucleoside overhang, and the secondary sequences contained either one or three copies of the same 18-mer sequence. The actual assembly consisted of the synthesis of the primary sequence: 5'-CAG-TCA-CTA-CGC-TTT-T-BM-T-TTT- GCG- TAG-linker-T-3',where linker = -O-CH_2CH_2-SO_2-CH_2CH_2-O- and T indicates the thymidine-CPG solid-support. The fragment was next detritylated, capped with acetic anhydride, and treated with 0.5 M hydrazine hydrate in pyridine/acetic acid (4:1 v/v) for 90 minutes. Chemical DNA synthesis was then continued with the secondary sequence: 5'-GAT-GTG-GTT-GTC-GTA-CTT-TTT-p-3' (for AutoLink-1), or 5'-(GAT-GTG-GTT-GTC-GTA-CTT)$_3$ -TTT-p-3' (for AutoLink-2). The structure of the AutoLink molecules is shown in Fig. 2.

Fig. 2. *Structure of AutoLink molecules.*

5'-(GAT-GTG-GTT-GTC-GTA-CTT)$_n$-TTT $\neg$|

T BM-TT-GCG-TAG-p-3'

T T CGC-ATC-ACT-GAC-5'

T T T

for AutoLink-1, n = 1 and for AutoLink-2, n = 3; $\neg$ indicates the N^4-(6-oxyhexyl) side-chain of BM.

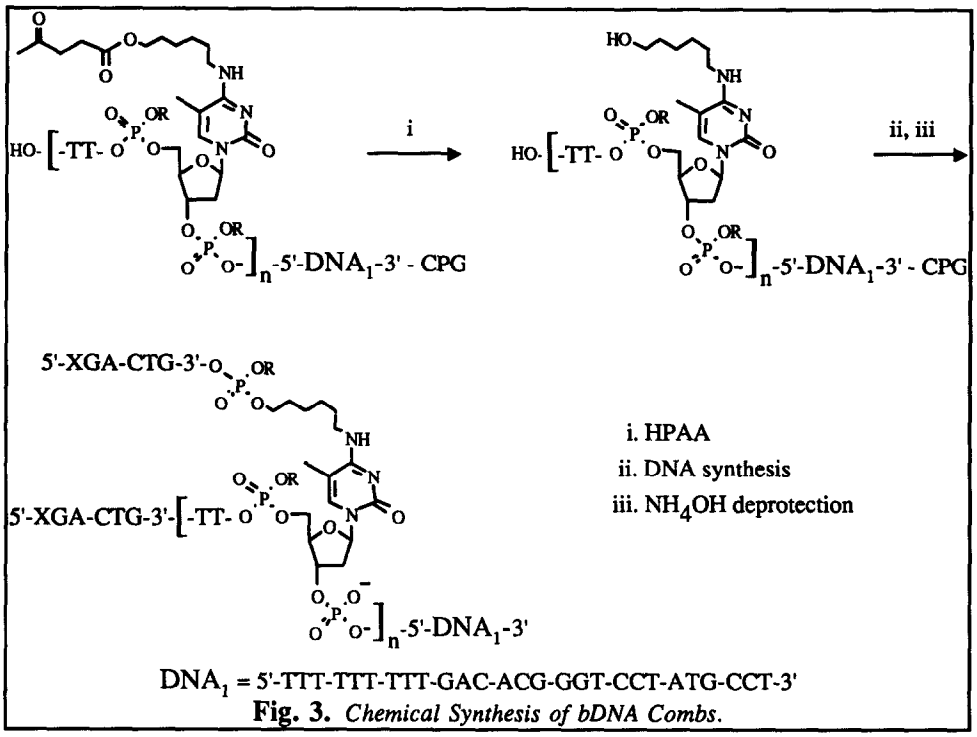

Fig. 3. *Chemical Synthesis of bDNA Combs.*

All oligomers were purified by PAGE. After gel elution the product was desalted on a Sep-Pak C-18 cartridge and ethanol precipitated.

Chemical ligation.

The following buffer solutions were used: buffer 1 (for EDC-induced ligations) - 50 mM MES, pH 6.0, 20 mM $MgCl_2$; buffer 2 (for BrCN-induced ligation) - 25 mM MES - $(C_2H_5)_3N$, pH 7.5, 20 mM $MgCl_2$.

EDC-induced ligation.

The mixture of linear and branched oligonucleotides in buffer 1 was cooled from 85° to 0° C in 2-3 hours, and then EDC in the same buffer was added to give a final concentration of 0.2 M. The nucleotide concentration was 3-5 mM (per monomer). The reaction mixture was incubated at 0° C in the dark. After 18 hours 3 M potassium acetate was added to give a final concentration of 0.3 M, and the oligonucleotide fraction was precipitated with 3 volumes ethanol. After drying the oligonucleotide fraction was analyzed by PAGE and HPCE.

BrCN-induced ligation.

The procedure for BrCN-mediated ligation was adapted from the method developed by Sokolova et al. [11]. The mixture of linear and branched oligonucleotides in buffer 2 was cooled from 85° to 0° C in 2-3 hours, and then a 5 M solution of BrCN in absolute acetonitrile was added (1/10 of the total volume). The nucleotide concentration was 3-5 mM (per monomer). After 3 minutes at 0° C potassium acetate (3 M) was added to a final concentration of 0.3 M, and the oligonucleotide fraction was precipitated with 3 volumes ethanol. After drying the oligonucleotide fraction was analyzed by PAGE and HPCE.

RESULTS AND DISCUSSION

The basic concept of chemical ligation has been extensively studied in model systems [4,5]. Two oligonucleotides with 3'-phosphate and 5'-hydroxyl termini, respectively, are brought in close proximity by hybridization with a complementary linker oligomer with which they both form a stable duplex. Thus the reactive groups are located in a nick in a double-stranded duplex and the effective concentration of the reactants is increased many orders of magnitude.

Two condensing reagents for chemical ligation have been studied, 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDC) and cyanogen bromide (BrCN). EDC-mediated introduction of natural phosphodiester linkages have proved to be very slow, requiring several days for completion [5]. Furthermore base modifications have been observed as a consequence of the long exposure to the coupling reagent. Substitution of the 5'-hydroxyl group for the more nucleophilic amino- or phosphate groups improved the efficiency of EDC-mediated ligation with yields of product containing a phosphoramidate or pyrophosphate linkage approaching 90-100% in a few hours [5].

Efforts to improve the slow EDC-mediated formation of the natural phosphodiester linkage in chemical ligations led to the development of alternative procedures using BrCN as the condensing reagent [12]. Sokolova et al.[13] found that BrCN without any additives was a very efficient coupling reagent in aqueous solution. The chemical ligation proceeded very rapidly and the reaction was virtually complete in a few minutes. They further noticed the absence of chemical modifications of the nucleobases. BrCN was, however, shown not to be suitable for the formation of linkages other than the natural phosphodiester [5]. Natural phosphodiester linkages were introduced with BrCN, whereas modified internucleotidic linkages (P-O-P or P-N) were synthesized using the EDC coupling reagent.

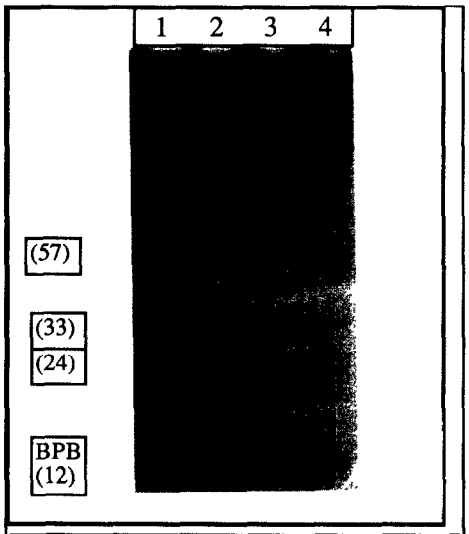

FIG. 4. *PAGE analysis of chemical ligation of linear oligonucleotides (10% gel, UV shadow). EDC-mediated phosphoramidate linkage formation (lane 1), and pyrophosphate linkage formation (lane 2); BrCN-mediated phosphodiester linkage formation (lane 3). Initial 33-, 24-, and 12-mer oligonucleotides (lane 4). The component ratio of 33-mer, 24-mer and linker was 1: 1.5 : 3. BPB indicates Bromophenol Blue marker.*

The efficiency of the chemical ligation depended upon the nature of the terminal nucleotides in the oligonucleotides to be joined [14]. The best contact, -G-3'-p + 5'-T-, was used throughout this study. The chemical synthesis of bDNA combs has been optimized with 6 nucleotide residues in the secondary sequence [15]. This type of bDNA comb was used in conjunction with a 12-mer linker, resulting in a 6+6 nucleotide overlap for stabilization of the duplex.

Chemical ligation reactions were initially conducted in a simple three-oligomer system in which a 24-mer terminating in G-3'-p (1) was ligated to a 33-mer terminating in 5'-XGA-CTG, where X = 5'-HO-T (2), 5'-NH_2-T (3), or 5'-p-T (4). The two oligomers were hybridized in the presence of the complementary 12-mer linker (5). The PAGE analysis of the test ligations are presented in Fig. 4. All the ligations produced the 57-mer product. The modified linkages were formed more efficiently than the natural phosphodiester linkage. The estimated coupling yields for the modified linkages were 95% for phosphoramidate (lane 1) and pyrophophate (lane 2), whereas phosphodiester formation using BrCN as coupling reagent proceeded in 90% yield (lane 3).

The linear 24-mer sequence terminating in G-3'-p (1) was next chemically ligated to a bDNA comb containing five secondary sequences (5x bDNA). The constructions used to assemble large bDNA molecules containing 5 or 15 branches by chemical ligation from bDNA combs and linear or AutoLink DNA molecules are shown in Fig. 5. The 5x bDNA molecule, which was the limiting component, contained the 6-mer secondary sequence 5'-XGA-CTG-3', where X = 5'-HO-T (7), 5'-NH_2-T (8), or 5'-p-T (9). After the predetermined ligation time the reaction mixtures were analyzed by PAGE and by HPCE. Starting 5x bDNA comb, 7 - 9 , was consumed in all cases and a series of slower migrating bands corresponding to the mono-, di-, tri-, tetra-, and penta-substituted bDNA molecule were produced. Fig. 6A and B show the PAGE results of the BrCN and the EDC-mediated reactions. Both of

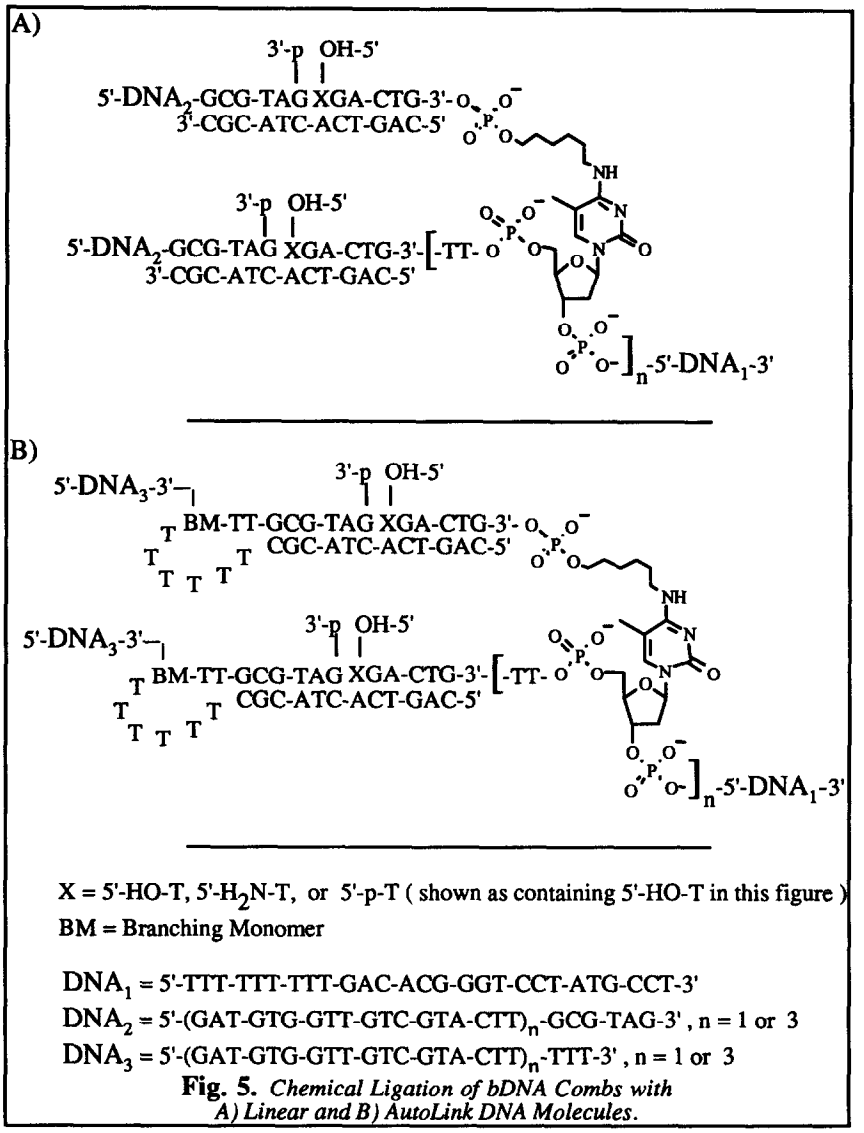

Fig. 5. *Chemical Ligation of bDNA Combs with A) Linear and B) AutoLink DNA Molecules.*

the modified linkages were formed with higher efficiency resulting in the tetra- and penta-substituted bDNA molecules preferentially. Formation of the pyrophosphate linked material (Fig. 6B, lane 2) was the most efficient. The HPCE profile of the EDC-mediated formation of pyrophosphate linked 5x bDNA is shown in Fig. 7. Quantitation of UV_{254} detected bands by HPCE analysis was used to estimate the coupling yields for each of the reactions mixtures (TABLE 1).

The most favorable product distribution was achieved in the pyrophosphate-linked product, where 39 % of the UV material was the fully derivatized 5x product. In contrast for the phosphoramidate-linked material the fully derivatized 5x product accounted for 19 % of the UV material. Formation of the natural phosphodiester

TABLE 1. Coupling Yields for Ligation Reacions of oligomer 1 with 5x bDNA combs.

5x bDNA	Linkage	[%] Substitution mono	di	tri	tetra	penta
7	O-P-O	20	33	31	12	2
8	O-P-N	2	9	31	39	19
9	O-P-O-P-O	0	2	17	43	39

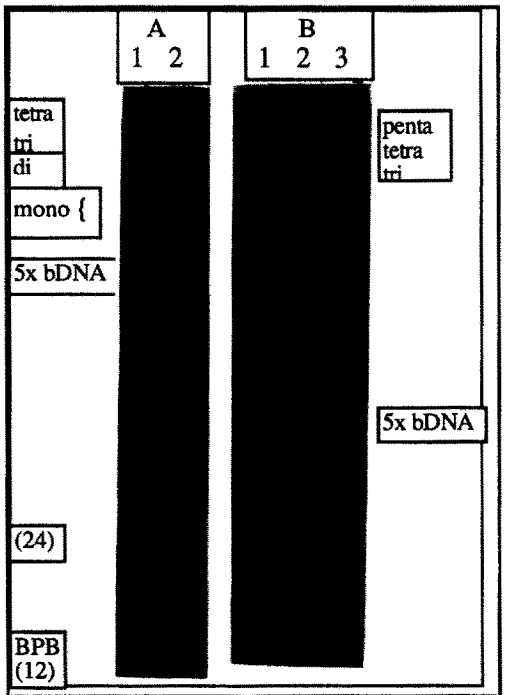

FIG. 6. *PAGE analysis of chemical ligation of branched DNA. A) BrCN-mediated phosphodiester linkage formation (lane 1), and mixture of 5x bDNA (7) and 24-mer (lane 2). B) EDC-mediated phosphoramidate linkage formation (lane 1), and pyrophosphate linkage formation (lane 2); 5xbDNA (8) (lane 3). The component ratio of 5x bDNA, 24-mer and linker was 1: 2: 3 (per each secondary sequence of comb). **Note**: the scales of mobility are different for A) and B); 5x bDNA is internal standard.*

linked product was less efficient.

In an effort to achieve more efficient ligations we developed a novel linker strategy in which the ligation linker was covalently attached to the incoming oligomer. The design involved a branched oligonucleotide in which the primary sequence could hybridize to form a stable, intramolecular hairpin and the 6-base 5' overhang acted as a linker. The desired linear oligomer was the secondary sequence. The structure of the molecule, Auto-Link, is shown in Fig. 2.

The kinetics of formation and the stability of the resulting duplex formed with the bDNA combs were expected to be improved over the three-piece system. Furthermore, the AutoLink molecule would assure that the

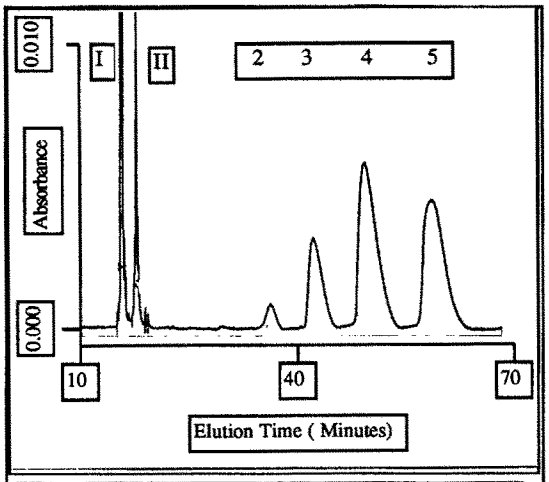

FIG. 7. *HPCE analysis of EDC-mediated pyrophosphate linkage formation. Peaks I and II correspond to the linker and 24-mer, respectively. Peaks 2, 3, 4, and 5 are the di-, tri-, tetra-, and penta-substituted reaction products, respectively.*

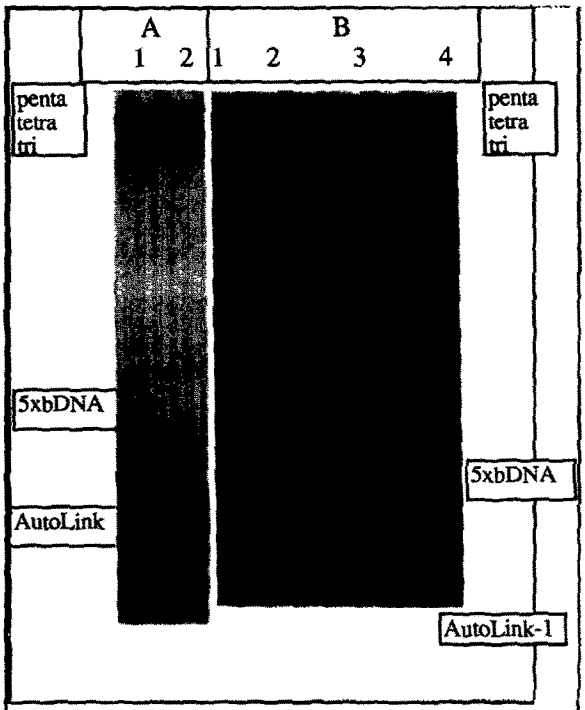

FIG. 8. *PAGE analysis of chemical ligation of branched DNA (10% gel, UV shadow). A) A mixture of 5x bDNA (7) and AutoLink-1 (lane 1); BrCN-mediated phosphodiester linkage formation (lane 2). B) 5x bDNA (9) (lane 1); AutoLink-1 (lane 2); EDC-mediated phosphoramidate linkage formation (lane 3), and pyrophosphate linkage formation (lane 4). The component ratio of 5x bDNA and AutoLink-1 was 1: 2 (per each secondary sequence of the comb).*

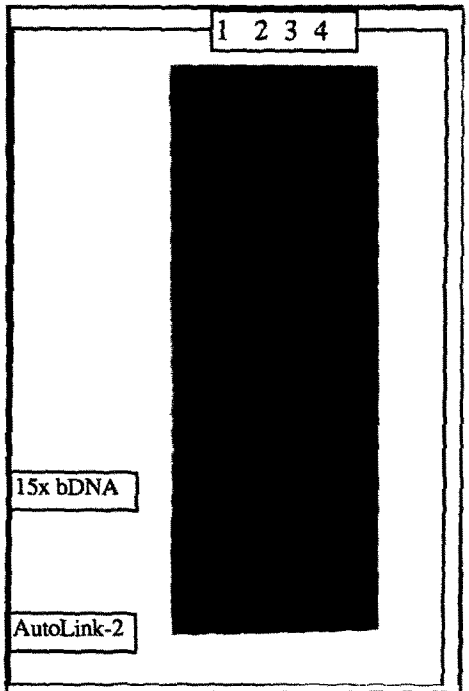

FIG. 9. *PAGE analysis of chemical ligation of branched DNA (5% gel, UV shadow). A mixture of 15x bDNA (10) and AutoLink-2 (lane 1). EDC-mediated pyrophosphate linkage formation, where component ratio of 15x bDNA and AutoLink-2 (per each secondary sequence of the comb) was 1: 0.5 (lane 2), 1: 1 (lane 3), and 1: 2 (lane 4).*

incoming oligomer and the ligation linker would always be present in equimolar concentrations.

AutoLink-1, containing 48 nucleotides, was tested in ligations involving the 5x bDNA combs 7 - 9, where AutoLink-1 replaced both the linear oligomer 1 and the linker 5. The BrCN-mediated reaction (Fig. 8A lane 2) resulted in the complete disappearance of the starting 5x bDNA comb. The main products were tri-, tetra- and penta-substituted bDNA in yields estimated to be 5, 55, and 40%, respectively, thus an improvement in the product distribution over the linear system. The effect of using AutoLink was even more pronounced in the formation of modified linkages. The PAGE analysis of the EDC-mediated ligations is shown in Fig. 8B. The main products were the tetra- and penta-substituted bDNA in estimated yields of 60 and 35%, respectively, for phosphoramidate linked material (lane 3), and 40 and 60%, respectively, for pyrophosphate linked material (lane 4).

These results encouraged us to incorporate AutoLink-2, a molecule in which the secondary sequence was extended to incorporate three copies of the 18-mer (containing a total of 85 nucleotides). It was tested in ligations with the 15x bDNA comb 10 in the presence of EDC. The molar ratio of 15x bDNA comb 10 / AutoLink-2 was varied from 0.5 to 2.0. Fig. 9 shows the PAGE analysis of these reactions. The starting

material was consumed in all cases. Increasing the ratio of AutoLink to 10 in the reaction mixtures produced a ladder of bands corresponding to all the possible products, each with an increasing number of long branches. The gel picture allowed the number of long branches in each species to be estimated: 1 to 6 (lane 2), 3 to 9 (lane 3), and >10 (lane 4). However, the 5% slab gel could not resolve the individual species with more than 10 AutoLink-2 arms. The main product(s) in these ligations were rather large bDNA molecules containing more than 1000 nucleotides.

The facile incorporation of modified linkages into bDNA molecules using chemical ligation suggests a convenient route to the characterization of these complex molecules. Phosphoramidate and pyrophosphate linkages are cleaved specifically under mild conditions without damage to the DNA oligomer with aqueous acetic acid and trifluoroacetic anhydride in aqueous pyridine, respectively [16]. After purification of the ligated bDNA molecule specific cleavage at the ligation sites and quantitation of the resulting linear fragments would yield the number of branches [15].

These preliminary experiments demonstrate that chemical ligation can be used in the construction of bDNA amplifier molecules. The presence of the covalently attached ligation linker of the AutoLink molecule and its unique hybridization characteristics greatly facilitated the construction of large bDNA amplification multimers. It is envisioned that it will be feasible to use chemical ligation in the construction of larger bDNAs and of complex objects based on DNA [17].

Chemical ligation in the construction of large multi-branched DNA oligomers is currently being used to assemble a fully functional amplifier containing 15 branches with a total of 45 secondary sequences for viral quantification [18].

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