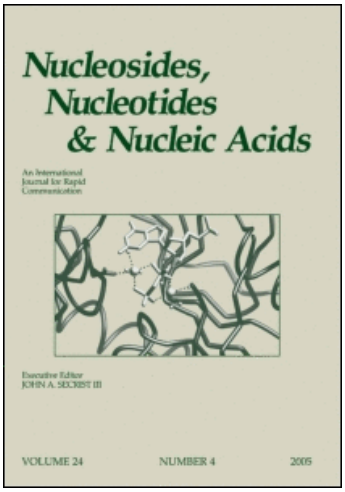

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Improved Methods for the Synthesis of Branched DNA (bDNA) for use as Amplification Multimers in Bioassays

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IMPROVED METHODS FOR THE SYNTHESIS OF BRANCHED DNA (bDNA) FOR USE AS AMPLIFICATION MULTIMERS IN BIOASSAYS.

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ABSTRACT: We have reported the synthesis of comb type bDNA where a unique, primary oligonucleotide fragment is tailed with several identical copies of a secondary oligonucleotide[1]. The strategy for synthesis of these molecules is based on the use of a nucleoside analog branching monomer. Recent improvements in solid phase methods enable the synthesis of bDNA with as many as 50 copies of secondary sequences. Characterization of these bDNA is also discussed.

Detection of infectious organisms that occur in very low numbers in vivo (such as HCV and HIV) has been a major challenge to biotechnology. Direct detection of viruses by immunological methods has been difficult or impossible. Although target amplification methods (such as PCR, LCR, TAS, and 3SR) have been successfully used for qualitative detection of small quantities of nucleic acids (yes/no answer), we have sought a signal amplification method that could be employed for quantitative low-level viral detection (if yes, how much answer). We have previously reported[2] the synthesis and application of oligonucleotide complexes (amplification multimers) that allow for the incorporation of multiple labels per probe-target nucleic acid complex. Amplification multimers composed of bDNA molecules that can bind 5 labels each have been employed to quantitatively detect as few as 60,000 HBV particles directly in human serum samples.

Our recent effort to synthesize larger bDNA for improved sensitivity has been concentrated on "comb" type molecules (Figure 1)[1] that have shown the most promising results thus far. These comb molecules are composed of two distinctive domains of oligonucleotide sequences: the "comb body" that consists of target specific fragment and branching monomers linearly arranged, and multiple "comb teeth" consisting of several identical sequences (to which labeled probes can hybridize) bound to the branching monomers.

In the solid-phase synthesis of comb molecules the body was assembled with standard phosphoramidite synthesis utilizing regular nucleoside phosphoramidites and a branching monomer containing an exocyclic hydroxyl function on a cytidine analog (Figure 2) protected with levulinate (primary synthesis). After completion of the primary

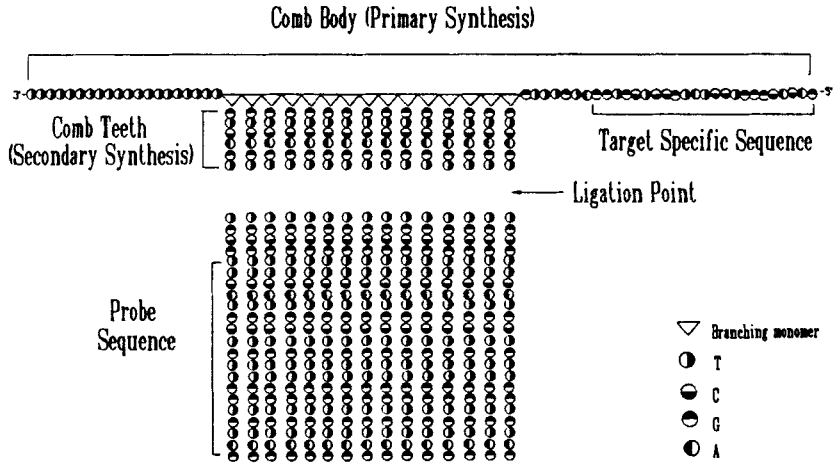

Fig. 1. "Comb"-type Branched DNA (bDNA)

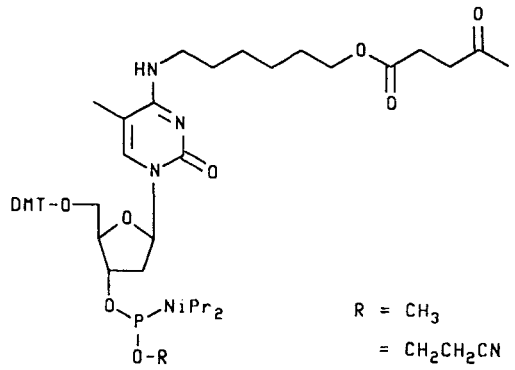

Fig. 2. Protected Comb Branching Monomer - The "BM" Molecule

sequence, the levulinate groups were specifically removed with hydrazine and the branch points were extended simultaneously to produce the comb teeth (secondary synthesis). Direct chemical synthesis of amplification multimers with less than 10 comb teeth containing less than 20 nucleotides each has proven possible; however, larger multimers with more than 15 teeth containing more than 60 bases each has only been possible with a combination of chemical synthesis and enzymatic ligation methods. These highly branched comb molecules were synthesized from a body with more than 15 branching monomers and relatively short side chains (less than 10 bases that act as enzymatic ligation points) on a solid support. After the comb molecules were cleaved from the support, the teeth were ligated in solution to a probe sequence to give the final amplification multimer.

The synthesis of highly branched comb molecules has been a tremendous challenge for the state-of-the-art solid phase oligonucleotide synthesis. Initially we encountered difficulties when attempting to introduce side chains using CPGs with 500 to 1000 A pore sizes. The secondary synthesis results were greatly improved when larger

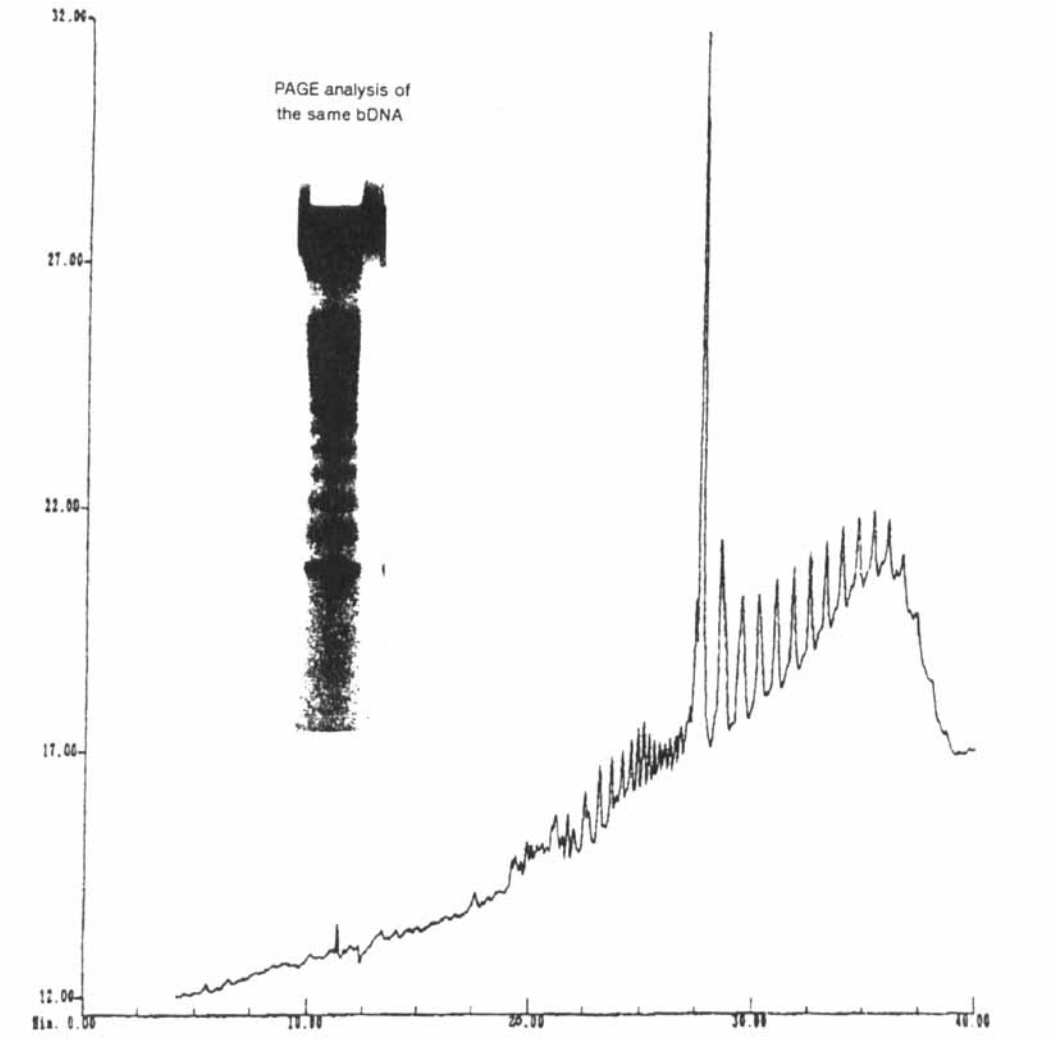

Fig.3. Gel-filled Capillary Electrophoretic Analysis of a Synthetic 15-site bDNA: 3'- $T_{20}(BM)_{15}[GTCAGT\text{-}5']_{15}GTTTGT$ -5'

pore size (2000-3000 A) were used suggesting that steric hindrance had previously limited our progress. Depending on the number of branching sites on a comb body, a single nucleotide extension of all the sites in the secondary synthesis could lead to a 50% increase in molecular size.

Since the branching sites are expected to be close to each other on the support, the diffusion of oligonucleotide synthesis reagents through the porous media becomes critical in order to achieve sufficient yields. Reaction rates can be facilitated by increasing either the reagent concentrations or the reaction times in various steps. We also noted that the removal of dimethyltrityl (DMT) cations from the support in secondary synthesis was very sluggish with the standard TCA/CH_2Cl_2 formulation, leading to poor results. This putative solvent effect was eliminated by using a mixed detritylation solvent system containing toluene and dichloromethane (1:1 v/v).

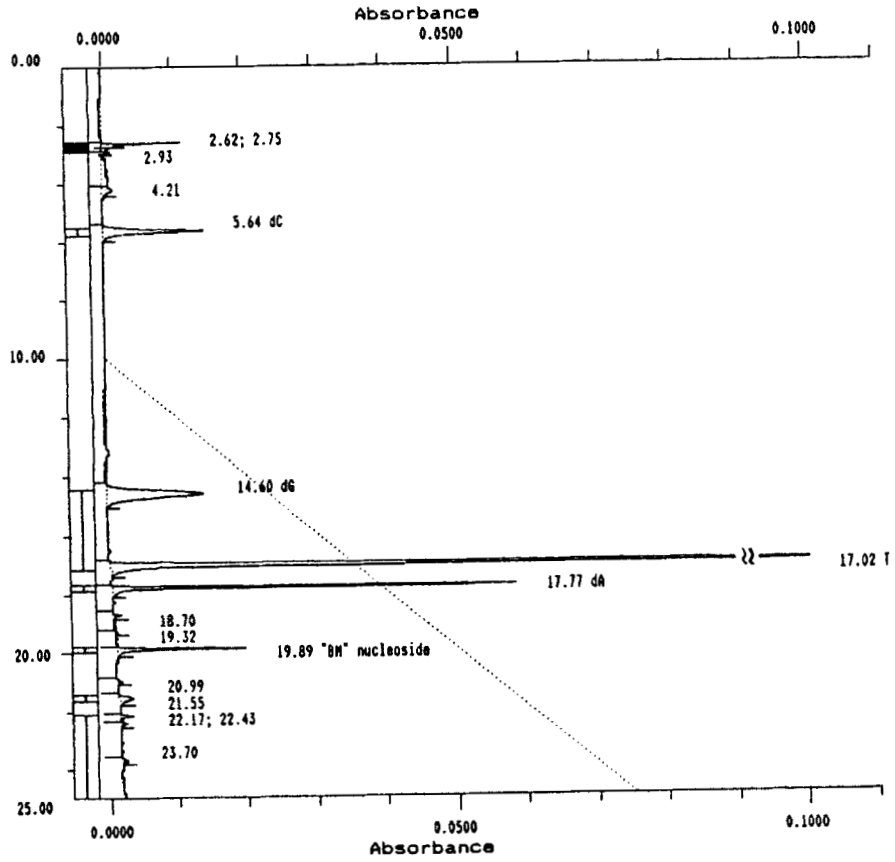

Fig.4. HPLC Analysis of an Enzymatically Degraded bDNA:
3'- $T_{40}(BM)_{15}[GTCAGT\text{-}5']_{15}GTTTGTGG$ -5'

Characterization of the comb molecules has proven to be a formidable task. The conventional polyacrylamide gel electrophoretic (PAGE) methods do not provide sufficient resolution. This problem was further complicated by retarded mobility of comb molecules through the polyacrylamide gel matrix due to their branched structure. However, the newly developed gel-filled capillary electrophoresis (Figure 3) seems to be a promising alternative separation technique.

A chemical degradation method was recently developed to assist in the structural analysis of comb molecules (see Horn, T. et al, this journal). In addition, the base composition of comb molecules can be determined by HPLC methods using standard enzymatic degradation protocols (Figure 4).

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