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Biophysical properties of modified oligodeoxynucleotides

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BIOPHYSICAL PROPERTIES OF MODIFIED OLIGODEOXYNUCLEOTIDES

by

Robin S. Quartin

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ABSTRACT

BIOPHYSICAL PROPERTIES OF MODIFIED OLIGODEOXYNUCLEOTIDES

by

Robin S. Quartin

Advisor: Dr. James G. Wetmur

The thermodynamic and biochemical properties of two types of modified oligodeoxynucleotides were investigated in order to improve upon current applications and to develop new molecular biology tools.

Oligodeoxynucleotides containing uncharged, chiral methylphosphonate linkages bound specifically to immobilized complementary DNA. These oligodeoxynucleotides were used to examine the effect of reduced charge on the thermodynamics of binding to complementary DNA or complementary oligodeoxynucleotides. The free energy decrement per linkage was –0.75 kcal/mol in high salt. The absence of a charge change for nearest neighbor base pairs containing a methylphosphonate linkage led to greater hybrid stability for substituted oligodeoxynucleotides at low salt. Finally, analysis of dissociation temperatures indicated that substitution of methylphosphonate linkages at high salt only affected the reverse rate constant.

Oligodeoxynucleotides with different arrangements of diester and methylphosphonate linkages were examined for nuclease sensitivity in vitro and in tissue culture using an assay for the ability to gel–shift a labeled

complementary phosphodiester oligodeoxynucleotide. Both 5' and 3' exonuclease function was impaired by methylphosphonate linkages. The smallest span of internal phosphodiester linkages correlated with the greatest resistance to endonuclease. However, in cell culture the half-lives of these oligodeoxynucleotides were independent of the number of contiguous phosphodiester linkages. RNase H assays indicated that a minimal span of three internal phosphodiester linkages in a methylphosphonate-substituted oligodeoxynucleotide was needed to allow cleavage of the RNA in the duplex.

We have also developed a highly sequence-specific capture reaction based on increased DNA-DNA hybrid stability due to substitution of bromodeoxycytidine (BrdC) for deoxycytidine (dC). BrdC-containing oligodeoxynucleotides displaced dC-containing strands from duplexes with blunt-ends or 3'-overhangs. A BrdC-containing oligodeoxynucleotide, used for transient sequence-specific invasion at a particular PstI site, was captured using DNA ligase and a linker oligodeoxynucleotide at over 300 times the rate for an unrelated PstI site. Incorporation of an incorrect nucleotide into a displacer strand demonstrated that branch migration terminated at a mismatch. A branched, BrdC-containing ligated capture reaction product was cloned and sequenced.

FORMAT OF THESIS

This thesis was prepared according to the guidelines of the City University of New York. There is a general introductory chapter, followed by three research article chapters, and an overall discussion chapter. Chapter II is a previously published article, chapter III represents unpublished work done in collaboration with Christine L. Brakel and Joanne P. Spadoro of Enzo Biochem, and chapter IV is an article that has been submitted for publication. Each research chapter contains an introduction, a materials and methods section, and research and discussion sections (combined in chapter II). References for all of the chapters are compiled in the bibliography.

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PUBLICATIONS

Quartin, R.S., Monestier, M., Moran, T.M., Waters, S.J., Fredrickson, G.G., and Bona, C.A., Characterization of the fine specificity and antigen markers associated with the receptor of autoreactive T-cell hybridomas. Cellular Immunology, 110, 163-175 (1987).

Quartin, R.S. and Wetmur, J.G., The effect of ionic strength on the hybridization of oligodeoxynucleotides with reduced charge due to methylphosphonate linkages to unmodified oligodeoxynucleotides containing the complementary sequence. Biochemistry, 28, 1040-1047 (1989).

Quartin, R.S., Plewinska, M. and Wetmur, J.G., Branch migration-mediated DNA cloning. (Submitted).

TABLES

FIGURES

I. INTRODUCTION

A. MODIFIED OLIGODEOXYNUCLEOTIDES

Oligonucleotides have been used as models for the study of the many complex interactions of nucleic acids with other nucleic acids (Breslauer, et al., 1986; Freier, et al., 1986) or nucleic acid binding proteins (Kadonaga & Tjian, 1986; Rauscher, et al., 1988). Oligodeoxynucleotides are important tools for sequencing (Gibbs, et al., 1989), mutagenesis (Kunkel, et al., 1987; Foss & McClain, 1987), polymerase chain reaction amplification (Saiki, et al., 1988), and other molecular biology techniques, and for antisense studies of various biological systems (van der Krol, et al., 1988), including therapeutic control of gene expression. Oligodeoxynucleotides possessing structural modifications exhibit qualitatively similar but quantitatively different interactions with complementary nucleic acids, proteins and other molecular compounds. The major theme in this thesis is the investigation of the thermodynamic and biochemical properties of modified oligodeoxynucleotides. A detailed understanding of the characteristic behavior of oligodeoxynucleotides with a particular type of modification provides a basis for the improvement of their current uses and for the development of new applications.

The two types of modifications addressed in this thesis are methylphosphonate substitution of the oligodeoxynucleotide backbone, and the replacement of deoxycytidine (dC) by the modified base, 5–bromodeoxycytidine (BrdC). The studies of methylphosphonate–substituted oligodeoxynucleotides have a bearing on the current use of these molecules as antisense agents.

The study of BrdC–substituted oligodeoxynucleotides has been expanded to include the development of a method for sequence–specific labeling and cloning.

B. OLIGODEOXYNUCLEOTIDES AS ANTISENSE AGENTS

The term "antisense" was initially used to describe the RNA involved in the regulation of messenger RNA translation and/or lifetime in various prokaryotic organisms (Green, et al., 1986). Naturally occurring antisense RNAs have been found in eukaryotes as well (Heywood, 1986; Stevens, et al., 1987). The artificial regulation of gene expression in eukaryotes by antisense RNA and by short, complementary sequences of oligodeoxynucleotides has been the subject of a recent review (van der Krol, et al., 1988).

There are a number of factors to be considered when using antisense oligodeoxynucleotides. The length of the oligodeoxynucleotide is important to both the specificity of the effect and to the stability of the hybrid to be formed. The binding of an oligodeoxynucleotide to complementary RNA is concentration dependent. The shorter the antisense nucleotide sequence, the more likely it is to find additional complementary sequences within the mRNA population in a cell, and hence the more proteins that may be affected at once. Longer sequences are more specific and also confer greater stability to the mRNA:oligodeoxynucleotide hybrid than shorter sequences. However, longer oligodeoxynucleotides have more difficulty penetrating cell membranes. Both delivery into the cell and stability to endo– and exonucleases in the cellular environment are important parameters.

Single–stranded oligodeoxynucleotides with standard phosphodiester backbones might not be expected to freely cross a cell membrane, and would certainly be targets for degradation by nucleases. Zamecnik and coworkers, however, have reported success in cell culture with an antisense 13–mer oligodeoxynucleotide which blocks Rous sarcoma virus replication (Zamecnik & Stephenson, 1978) and with a 20–mer which inhibits human immunodeficiency virus (HIV) replication and expression (Zamecmik, et al., 1986). It is interesting to note that a better antisense effect was detected with oligodeoxynucleotides that were blocked at the 3'– and 5'–terminal hydroxyls with isourea derivatives (Zamecnik, et al., 1986). More recently, by testing a panel of standard oligodeoxynucleotides targeted to a large number of regions of the HIV RNA, the terminal repeats, the primer binding site, and certain splice acceptor sites were most effectively blocked (Goodchild, et al., 1988). The results of a study of oligodeoxynucleotide stability in cell culture suggested that unmodified oligodeoxynucleotides would be rather stable in reticulocyte lysate and cell culture systems but would not be very effective in a whole animal experiment (Wickstrom, 1986).

In the interest of generating the highest degree of efficiency in translation inhibition, it is desirable to chemically alter or augment the oligodeoxynucleotide in order to facilitate its entry into the cell and/or increase its half–life, while maintaining or increasing its affinity for the specific mRNA. One type of modification to increase affinity (and provide some nuclease protection) is the coupling of the oligodeoxynucleotide to an agent that can intercalate between the oligodeoxynucleotide and the mRNA. Oligodeoxynucleotides that are covalently linked at the 3' end to acridine

derivatives have been used to inhibit the synthesis of the gene 32–encoded protein of bacteriophage T4, in vitro (Toulmé, et al., 1986). These oligodeoxynucleotides provided better efficiency of inhibition than homologous unsubstituted oligodeoxynucleotides. However, the contribution of the intercalating agent to the duplex stability decreased as the length of the oligodeoxynucleotide chain is increased.

The presence of 3' or 5' covalently–linked acridine moieties protected the oligodeoxynucleotide from exonucleases, but not endonucleases (Toulmé, et al., 1986). Oligo(alpha–deoxynucleotides) synthesized from alpha anomers of thymidine, rather than the native beta anomer, demonstrated greater nuclease resistance, form stable duplexes with polyribonucleotides, and were also able to be linked to acridine or other derivatives (Thuong, et al., 1987).

Oligodeoxynucleotides with phosphorothioate linkages are DNA analogues originally designed for the study of the interactions between DNA and enzymes (Eckstein, 1985). The altered backbone structure due to the sulfur groups conferred resistance to degradation by certain nucleases. Phosphorothioate–substituted oligodeoxynucleotides are also diasteriosomeric and have decreased affinity for their complements. This has been found to be more so with AT base pairing than for GC (Stein, et al., 1988). In an antisense study to inhibit HIV infection of cell cultures, the results were consistent with a mechanism of HIV inhibition involving a non–sequence–specific interaction of the oligodeoxynucleotides with their target, possibly the reverse transcriptase (Matsukara, et al., 1987). Homopolymeric oligophosphorodithicates have recently been shown to be highly effective inhibitors of HIV reverse transcriptase (Caruthers, et al., 1989).

Improved delivery of antisense oligodeoxynucleotides into cells can be achieved by conjugation to poly(L–lysine). Such conjugates have been used at relatively low concentrations to efficiently inhibit the synthesis of vesicular stomatitis virus (VSV) proteins and VSV activity in cell culture (Lemaitre, et al., 1987). The poly(L–lysine) facilitated the entry and survival of the oligodeoxynucleotide in the cell by a mechanism that has not been established.

C. THE METHYLPHOSPHONATE LINKAGE IN ANTISENSE STUDIES

Methylphosphonate oligodeoxynucleotides are DNA analogues which contain a modified backbone structure where the negatively charged phosphodiester linkage has been replaced by an uncharged methlyphosphonate (Miller, et al., 1985). The modifications are incorporated during the synthesis of the oligodeoxynucleotide, originally through manual coupling of protected nucleoside 3'–(methylphosphonic imidazolide) monomers (Miller, et al., 1986), and more recently, by automation of phosphonamidite chemistries with methylphosphonamidite monomers. Methylphosphonate linkages are chiral, and exist in two different isomeric configurations: pseudoaxial (S) and pseudoequatorial (R) (Miller, et al., 1979). This results in multiple diestereoisomers for each sequence that is synthesized, but does not seem to adversely affect its ability to base pair with complementary nucleic acids (Miller, et al., 1981). The question of sequence specificity of a fully–modified methylphosphonate oligodeoxynucleotide is addressed in chapter II.

Various characteristics of methylphosphonate oligodeoxynucleotides make them particularly amenable to antisense studies in cell culture and in vivo. The non-charged linkages render methylphosphonates quite lipophilic, allowing passage through the plasma membrane. In addition, the altered form of the backbone is a structure resistant to various nucleases, so the oligodeoxynucleotide has a long half-life in cell culture medium and inside cells (Miller, et al., 1981). A methylphosphonate octamer, complementary to the acceptor splice junction of two immediate early mRNAs in HSV-1, was able to inhibit virus growth, early in the replicative cycle (Smith, et al., 1986). The highest concentrations of oligodeoxynucleotide used in the study (150 μM) gave the best inhibition of viral protein synthesis and viral activity, but there was a small degree of inhibition of synthesis of certain low molecular weight cellular proteins. This is attributable to increased favorability of partial duplex formation at higher oligodeoxynucleotide concentrations, and to the fact that such relatively short sequences are likely to find complements within the mRNA population of the cell. In a second study, methylphosphonate oligodeoxynucleotides complementary to the initiation codon region of the mRNA of some of the proteins of VSV were able to decrease the synthesis of all VSV proteins in infected cells (Agris, et al., 1986). Factors contributing to this are the regions of sequence homology among the various viral mRNAs, interactions of oligodeoxynucleotides with replication intermediates causing a decrease in the minus RNA templates for mRNA, and the tight relationship of genes and proteins in the viral infective cycle.

Several modified forms of oligodeoxynucleotides were compared for their ability to inhibit expression of the plasmid-directed chloramphenicol

acetyltransferase gene in transfected cells (Marcus–Sekura, et al., 1987). Inhibition with the methylphosphonate oligodeoxynucleotide was dependent on both the concentration and chain length. A phosphorothioate oligodeoxynucleotide was about twice as effective as its methylphosphonate analogue, which was about two times better than the normal oligodeoxynucleotide. In another study, which involved the inhibition of HIV–directed syncytia formation and p24 expression, antisense effectiveness of fully–substituted methylphosphonate oligodeoxynucleotides was also concentration and length dependent (Sarin, et al., 1988). A 21–mer with two consecutive methylphosphonates at the 3' and 5' ends was no more effective than the control 20–mer diester. A comparative study of the ability of standard phosphodiester and methylphosphonate–substituted oligodeoxynucleotides to block the translation of human dihydrofolate reductase in a reticulocyte lysate system found that while fully–modified methylphosphonates bound selectively to the target RNA, they did so with 275–fold lower affinity than their respective phosphodiester analogues (Maher & Dolnick, 1988).

D. THERMODYNAMICS

A major part of the work presented in chapters II and IV involves the study of the thermodynamic behavior of oligodeoxynucleotides with methylphosphonate or BrdC substitutions, respectively. Results of studies of the affinities of natural, diester forms of nucleic acids for complementary single–stranded nucleic acids have been compiled (Breslauer, et al., 1986; Freier, et al., 1986). In this thesis, methylphosphonate and BrdC modifications

were examined for their effects on nucleic acid hybridization in an effort to define the conditions for stable duplex formation with complementary nucleic acid sequences.

1. METHYLPHOSPHONATE THERMODYNAMICS

Methylphosphonate dinucleotides were shown to form stable 1:2 complexes with polydeoxy– and polyribonucleotides and to dissociate from these complexes at higher temperatures than corresponding phosphodiesters (Miller, et al., 1979). Such results are due to the lower charge repulsion between the non–ionic methylphosphonate linkage and the negatively–charged diesters on the complementary dinucleotides. However, it was shown that singly–substituted, self–complementary octanucleotides formed less stable duplexes than their respective diester analogues (Bower, et al., 1987). The dinucleotide studies were done in very low salt conditions (10mM Tris, 10mM $MgCl_2$) while the octamer studies were carried out at a range of 0.05 to 1.0M NaCl. The methylphosphonate linkage has a complex effect on nucleic acid hybridization. While the presence of the non–ionic methylphosphonate linkages results in lower charge repulsion within a duplex (or triplex, in the above case), there are steric effects due to the fact that a methyl group has replaced an oxygen. This results in a degree of destabilization to a duplex for every incorporated methylphosphonate. This effect is compounded by the isomeric forms, particularly in multiply–substituted oligodeoxynucleotides. In both of the studies described above, the two isomeric forms were examined separately, and the R type was found to allow for more stable base–pairing

than the S type. One of the aims of the work presented in chapter II was to derive the average value for the change in free energy per methylphosphonate linkage which relates to the destabilizing effect.

The coil (single–stranded) form of DNA has a lower charge density than the helical (double–stranded) form (Cantor & Schimmel, 1980). This is because in a DNA duplex the bases are more stacked than in the coil form, causing the negatively–charged phosphates to be closer to each other in the helix form. The greater repulsive forces in the helical form result in a greater association with positive ions. Therefore, there is a change in total charge between the helix and the coil forms. In this thesis a comparison is made between the effect of methylphosphonate and diester linkages on the change in charge density for this transition. A major focus in chapter II is an examination of the conditions under which methylphosphonate–substituted oligodeoxynucleotides are more stable than standard analogues in a duplex with their complement.

2. BROMODEOXYCYTIDINE THERMODYNAMICS

Early experiments have shown that the substitution of bromine at position C5 of pyrimidines leads to increased duplex stability. Radding et al. (1962) showed that dG–BrdC was a more thermally stable base pair than dG–dC. In another study, poly dI:poly BrdC had a T_m 26°C higher than that of poly dI:poly dC (Inman & Baldwin, 1964), and it was further shown that poly BrdC displaced poly dC from the poly dI:poly dC duplex to form a new duplex with poly dI (Inman, 1964). In chapter IV we examine the pH–dependent effect

of BrdC–substitution on oligodeoxynucleotide melting and derive the value of the change in free energy per incorporated BrdC nucleotide and the relative stability of a BrdC–dG base pair versus a dC–dG base pair.

E. BIOCHEMISTRY OF METHYLPHOSPHONATES

Questions concerning the biochemical nature of methylphosphonate–substituted oligodeoxynucleotides are addressed in chapter III. Of particular interest is the arrangement of phosphodiester and methylphosphonate linkages in an oligodeoxynucleotide that will promote both nuclease resistance and antisense activity.

1. NUCLEASE SENSITIVITY

Few studies of nuclease sensitivity of methylphosphonates have been done. Miller and coworkers (1981) incubated fully modified oligodeoxynucleotides, containing ^{3}H–thymidine in the 3' position, with mammalian cells in culture. After 18 hours 70% of the labeled thymidine recovered from cell lysates was associated with full–length oligodeoxynucleotides. Their results also suggested passive diffusion across the cell membrane as the mechanism of entry.

Agrawal and Goodchild (1987) used HPLC to examined the degradation of oligodeoxynucleotides containing methylphosphonate linkages by spleen and snake venom phosphodiesterases. In our studies we used a gel shift assay where hybridization to a ^{32}P–labeled, complementary oligodeoxynucleotide was

used to monitor the action of specific endonucleases and exonucleases on partially methylphosphonate–substituted oligodeoxynucleotides. This assay was also used to examine the stability of methylphosphonate oligodeoxynucleotides in various tissue culture conditions.

2. RNASE H

It has been shown that, in the wheat germ cell–free translation system, digestion of the RNA strand of the oligodeoxynucleotide:mRNA duplex by RNase H is the primary mechanism by which translation is blocked, rather than by physical impediment to the ribosomes (Minshull & Hunt, 1986). In fact, in one study using acridine–linked oligodeoxynucleotides (Cazenave, et al., 1987) and in another using unmodified oligodeoxynucleotides (Dash, et al., 1987), the complementary mRNA was found to be degraded by RNase H activity within cells. This may be the predominant mechanism by which antisense activity is effected by oligodeoxynucleotides in cells where RNase H is relatively abundant, and perhaps in such cells an antisense oligodeoxynucleotide may be more efficient than an antisense RNA. Fully methylphosphonate–substituted oligodeoxynucleotides, however, do not serve as RNase H substrates when hybridized to complementary RNA (Maher & Dolnick, 1988). We examined various partially methylphosphonate–substituted oligodeoxynucleotides for their ability to promote RNase H cleavage of complementary RNA transcripts. We were able to find compositions of partially methylphosphonate–substituted oligodeoxynucleotides which simultaneously displayed resistance to both endo– and exonucleases and

formed substrates for RNase H.

F. NEW TECHNOLOGY – SEQUENCE–SPECIFIC LABELING AND CLONING

The work in chapter IV concerns the development of a new method for sequence–specific labeling and cloning. The method employs BrdC–substituted oligodeoxynucleotides to displace one strand of a duplex by the mechanism of homologous strand exchange at a branch point (branch migration).

There are numerous examples of homologous strand displacement. The replication of the supercoiled, circular genome of mammalian mitochondria is initiated unidirectionally with the formation of a displacement loop (D–loop) (Robberson, et al., 1972). D–loop formation has also been demonstrated in vitro (Holloman, et al., 1975). The uptake of a large, complementary restriction fragment of single–stranded DNA by a supercoiled plasmid is driven entropically by the energy stored in the negative supercoils (Beattie, et al., 1977) and is analogous to a step in generalized recombination with branch migration (Radding, et al., 1977). Release of the superhelical free energy by nicking the supercoil results in rapid loss of the single–stranded DNA due to a directed branch migration process driven by the difference in entropy between the constrained single–strand of the D–loop and the released, unconstrained single–stranded DNA. A similar reaction has been demonstrated where a short strand was displaced from a linear duplex, presumably through a D–loop intermediate (Green & Tibbetts, 1981). Recently, RecA protein, using ATP as an energy source, was employed to reverse DNA branch migration reactions,

permitting the formation of D–loops in linear molecules (Rigas, et al., 1986).

R–loop formation, which is favored in 70% formamide, results when an RNA strand hybridizes to one strand of a DNA duplex and displaces the other (Thomas, et al., 1976). The reaction can be reversed by removing the formamide. The displacement of the RNA in an RNA:DNA hybrid by single–stranded DNA is the basis of a homogeneous nucleic acid hybridization assay, which detects ribonuclease–digestion products by chemiluminescence (Vary, 1987).

In our procedure a BrdC–substituted "displacer" oligodeoxynucleotide and a partially complementary "linker" oligodeoxynucleotide are captured at the end of a restriction fragment of DNA by the action of DNA ligase. With this technique a particular DNA fragment of interest can be labeled for detection without blotting and hybridization procedures, attached to a marker for affinity chromatography, or engineered to have a new 3' or 5' end to facilitate cloning. As a model for the development of this branch migration–mediated technique, experiments were also carried out to determine the rates at which BrdC–containing oligodeoxynucleotides displace their dC–containing analogues from duplexes with blunt ends and with overhangs. The increased thermodynamic stability of BrdC–dG base pairs compared to dC–dG base pairs is the basis for the high specificity of the technique.

II.

THE EFFECT OF IONIC STRENGTH ON THE HYBRIDIZATION OF OLIGODEOXYNUCLEOTIDES WITH REDUCED CHARGE DUE TO METHYLPHOSPHONATE LINKAGES TO UNMODIFIED OLIGODEOXYNUCLEOTIDES CONTAINING THE COMPLEMENTARY SEQUENCE

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A. INTRODUCTION

Oligodeoxynucleotide analogues containing modifications in their backbones are of particular interest for applications where nuclease resistance is important. For example, phosphorothioate deoxynucleotides have been used to examine the function of various enzymes and proteins that act on or associate with DNA (Eckstein, 1985), and oligophosphorothioate deoxynucleotides have been tested for antisense activity in vivo (Marcus–Sekura, et al., 1987; Matsukara, et al., 1987). In addition to being nuclease resistant and exhibiting antisense activity (Miller, et al., 1985; Murakami, Blake and Miller, 1985; Marcus–Sekura, et al., 1987; Matsukara, et al., 1987), oligodeoxynucleotide analogues containing non–ionic methylphosphonate linkages have decreased overall charge compared to their phosphodiester or phosphorothioate counterparts. The purpose of the work presented here with methylphosphonate–substituted oligodeoxynucleotides was to examine the effect of charge reduction on the thermodynamic behavior of helix–coil transitions of DNA.

The study was complicated by the chiral methylphosphonate bonds which exist in two isomeric configurations (S: pseudoaxial; R: pseudoequatorial) (Miller, et al., 1979). Helix–coil transitions of self–complementary, singly substituted R and S methylphosphonate oligodeoxynucleotides have been investigated (Bower, et al., 1987). These diastereoisomeric forms differed in ability to hybridize to a complementary DNA sequence, bonds of the R type contributing to more stable base–pairing than those of the S type. In spite of these complications due to chirality, methylphosphonate linkages were chosen for study because they are more

efficiently synthesized than non–chiral linkages such as carbamates (Stirchak & Summerton, 1987) and because no studies have been reported concerning the biological properties of oligodeoxynucleotides with non–chiral linkages.

For this study, several standard phosphodiester as well as one fully (10 of 11 linkages) and several partially methylphosphonate–substituted oligodeoxynucleotides were synthesized. All studies involved hybridizations of these oligodeoxynucleotides to complementary phosphodiester oligodeoxynucleotides which extended beyond the duplex region in both the 3' and 5' directions, just as is the case for hybridization to single–stranded DNA. The fully substituted oligodeoxynucleoside methylphosphonate was shown to be specific for its phosphodiester complement by Southern hybridization analysis.

Using known thermodynamic data for heteropolymeric DNA, natural DNAs and oligodeoxynucleotides, a single set of nearest neighbor thermodynamic parameters was obtained which fit the helix–coil transition for all oligodeoxynucleotides, including those containing adjacent guanosine residues. These results provided a quantitative framework for assessing the effect of the 3' and 5' tails on helix stability as well as the effect of methylphosphonate linkages at all ionic strengths on helix stability. Concentration–dependent melting temperatures for phosphodiester and partially methylphosphonate–substituted oligodeoxynucleotides were measured optically as a function of ionic strength. A gel migration assay was employed for studying duplex formation by the fully methylphosphonate–substituted oligodeoxynucleotide. Conditions for preferential binding of methylphosphonate–substituted oligodeoxynucleotides to single–stranded DNA are described. Finally, for each duplex type, the ionic strength dependence of

the time–dependent dissociation temperature was determined by examining elution from dot blots. These results, together with the thermodynamic results, indicate that substitution of methylphosphonate linkages at high salt only affects the reverse rate constant.

B. MATERIALS AND METHODS

1. OLIGODEOXYNUCLEOTIDES AND PLASMID DNA

All oligodeoxynucleotides (Table 1) were synthesized on an Applied Biosystems Model 380B DNA Synthesizer. Phosphodiester linkages were generated by standard phosphoramidite chemistry, and methylphosphonate bonds were introduced by the coupling of methylphosphonamidite monomers. Hydrolysis of base–protecting groups and cleavage from the support for phosphodiester oligodeoxynucleotides was accomplished by NH_4OH treatment, which was followed by ethanol precipitation. Fully and partially methylphosphonate–modified oligodeoxynucleotides were released and deprotected in ethylenediamine:ethanol (1:1) for 7 hours at room temperature (Miller, et al., 1986). Use of this alternate deprotection method with a phosphodiester oligodeoxynucleotide led to a product which was indistinguishable from that produced using NH_4OH deprotection. The methylphosphonate oligodeoxynucleotide containing the single 5' phosphodiester linkage was purified by NH_4HCO_3 elution from a DEAE–cellulose column (Miller, et al., 1986), and the oligodeoxynucleotides containing mixed phosphodiester and methylphosphonate bonds were purified by 4M ammonium acetate elution from NACS Prepac columns (Bethesda Research

Labs). All oligodeoxynucleotides were 5'–labeled with ^{32}P using T4 polynucleotide kinase, subjected to polyacrylamide gel electrophoresis on 20% acrylamide–8M urea gels, and visualized by autoradiography. The unmodified oligodeoxynucleotides had the correct mobility for their sizes. Oligodeoxynucleotides with methylphosphonate substitutions have lower mobilities than their phosphodiester analogues. Both fully and partially substituted oligodeoxynucleotides were predominantly one large product. Minor, shorter, presumably failure and/or cleavage sequences could also be detected. Plasmid pALA–D is a pUC9 expression vector containing the cDNA sequence of human δ–aminolevulinic acid dehydratase (ALA–D), a heme biosynthetic enzyme (Wetmur, et al., 1986).

2. SOUTHERN HYBRIDIZATION

The Southern (1975) blot was prepared according to a standard procedure (Maniatis, et al., 1982), and the Zetabind filter (AMF Cuno) was treated as recommended by the manufacturer. A two–hour prehybridization was carried out in 5X SSC (0.15 NaCl, 0.015M sodium citrate, pH 7.0), 10X Denhardt's solution (1% of each: ficoll, polyvinylpyrrolidone, and bovine serum albumin), 500 μg/ml sonicated and boiled salmon sperm DNA, and 0.05M sodium phosphate buffer, pH 7. The hybridization solution contained 5X SSC, 1% Denhardt's, 100 μg/ml salmon sperm DNA, and 0.02M sodium phosphate buffer, pH 7. The oligodeoxynucleotide was 5'–labeled with ^{32}P using T4 polynucleotide kinase, and added to the hybridization solution at a concentration, based on A_{260}, of 5–10 ng/ml. Hybridization was carried out as is described for the dot–blot analyses.

3. DOT BLOT ANALYSES

Dots of plasmid DNA were made by spotting 0.5 μg quantities of plasmid pALA–D onto Zetabind filters, drying at room temperature, denaturing 15 minutes in 0.5M NaOH, 1.5M NaCl, and neutralizing 15 minutes in 1M Tris–Cl (pH 8.0). Once dried at room temperature, filters were washed in 0.1X SSC, 0.5% SDS (sodium dodecyl sulfate) at 65°C for one hour to decrease the background during hybridization. The filters were prehybridized for 2–3 hours at 37°C in the same solution described for the Southern hybridization. ^{32}P–labeled oligodeoxynucleotide was added to the hybridization solution (also described above) at concentrations ranging from 4 to 20 ng/ml. These conditions facilitate dot–blot hybridization for methylphosphonate oligodeoxynucleotides. Hybridization was carried out at 37°C for 1/2 hour, and then at room temperature for 1.5 hours. Each filter was washed at the specified salt and temperature for 30 minutes.

4. MELTING TEMPERATURE ANALYSES

Complementary oligodeoxynucleotides were mixed 1:1 in various concentrations of salt: 6X SSC; 100mM, 20mM, or 2mM NaCl in 2mM Tris–Cl, 0.2mM EDTA. Solutions were heated at 0.3°C/minute in a one centimeter quartz cuvette in a Beckman Model 25 spectrophotometer equipped with water–jacketed cell holder. Temperature was monitored using a thermistor attached to the cell holder. Hyperchromicities for all 14–mer plus 18–mer duplexes were 21%. The concentration–dependent melting temperatures (T_m = t_m + 273.16) were calculated according to the method of Marky and Breslauer

(1987). T_m values reported are reliable to ±1°C.

5. GEL MIGRATION ANALYSES

The phosphodiester recipient oligodeoxynucleotide 18–P3 was 5'–labeled with ^{32}P using T4 polynucleotide kinase and purified using a spun column (Maniatis, et al., 1982) of Sephadex G–50 in NTE (100mM NaCl, 10mM Tris–Cl, 1mM EDTA), TE/5 (2mM Tris–Cl, 0.2mM EDTA). To determine binding stoichiometry for the fully–substituted oligodeoxynucleotide 12–Me^{10}, it was mixed with 5'–labeled complementary oligodeoxynucleotide 18–P3 at various ratios. Annealing was carried out at room temperature for at least 15 minutes prior to electrophoresis. To examine the stability of the 12–Me^{10} plus 18–P3 duplex under the conditions used for electrophoresis, the complex was formed at a stoichiometric ratio, cooled to 4°C and incubated with 12–P1 as a competitor. To measure competition of 12–P1 and 12–Me^{10} for 18–P3 at various salt concentrations under equilibrium conditions, both 12–mers were added to the 18–mer, incubated at 70°C for 5 minutes and cooled slowly to room temperature. Samples were loaded onto a 20% acrylamide gel in either 10% glycerin plus bromophenol blue and xylene cyanol or 2.5% ficoll 400. Gel electrophoresis was performed at 4°C at 250–400 volts, 5–15 milliamps in TBE (89mM Tris–Cl, 89mM borate, 1mM EDTA) buffer. The gels were dried and the results were obtained by autoradiography.

C. RESULTS AND DISCUSSION

All of the oligodeoxynucleotides used in this study are listed in Table 1.

TABLE 1: Oligodeoxynucleotides

RECIPIENT		COMPLEMENT	
#	SEQUENCE	#	SEQUENCE
18–P1	GGA TGC AGC TAA GTC AAG	14–P1	TGA CTT AGC TGC AT
		14–A	TG**A** CTT **A**GC TGC **A**T
		14–C	TGA **C**TT AG**C** TG**C** AT
18–P2	CTT GAC TTA GCT GCA TCC	14–P2	ATG CAG CTA AGT CA
		14–G	AT**G** CA**G** CTA A**G**T CA
		14–T	A**T**G CAG C**T**A AG**T** CA
18–P3	CGC CAT GCA GCC CCA GTC	12–P1	TGG GGC TGC ATG
		12–Me[10]	T**GG GGC TGC AT**G
No Recipient		12–P2	CAT GCA GCC CCA

N = BASE WITH METHYLPHOSPHONATE LINKAGE TO 3' SIDE

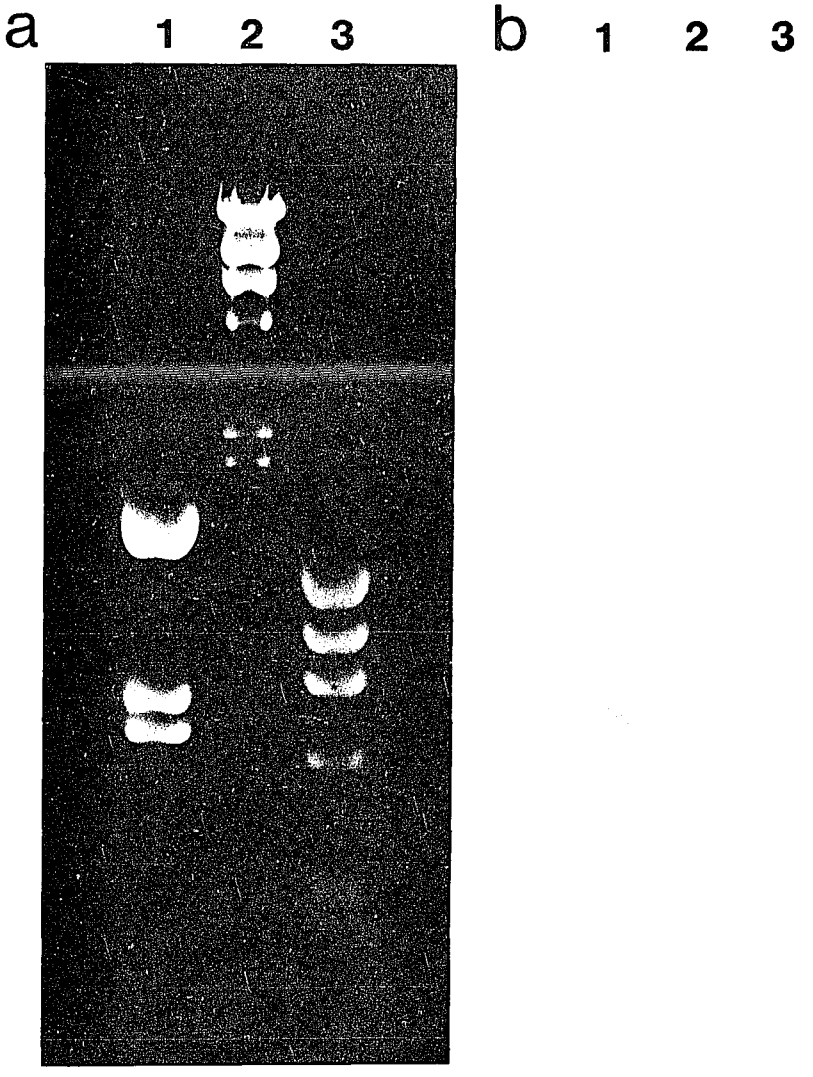
There are three "recipient" oligodeoxynucleotides, each of which is an 18–mer with a phosphodiester backbone. Oligodeoxynucleotide 18–P3 is complementary to the non–coding strand of the ALA–D gene (Wetmur, et al., 1986) in the region of the start of the protein sequence, corresponding to the cDNA positions of –4 to +14. Oligodeoxynucleotides 18–P1 and 18–P2 are complementary to each other and correspond to the cDNA positions of 627–644. For the purposes of this study, the use of sequences in the human ALA–D cDNA was fortuitous.

Each recipient oligodeoxynucleotide has a set of complementary oligodeoxynucleotides, identical in length and sequence, but differing in backbone structure. 12–mer sequences complementary to 18–P3 include a phosphodiester sequence (12–P1) and a sequence containing 10 methylphosphonate bonds and 1 phosphodiester bond at the most 5' position (12–Me^{10}). 14–mer sequences complementary to either 18–P1 or 18–P2 included phosphodiester sequences (14–P1 and 14–P2) and sequences synthesized employing one methylphosphonamidite (14–A, C, G, or T). The strand to be synthesized was dictated by a requirement to scatter the methylphosphonate bonds throughout the oligodeoxynucleotide. All the complementary oligodeoxynucleotides were shorter than the recipient oligodeoxynucleotides with duplex formation resulting in equal–length single–stranded 3'and 5' recipient dangling ends.

1. SPECIFICITY OF BINDING OF METHYLPHOSPHONATE OLIGODEOXYNUCLEOTIDES

In order to demonstrate the degree of specificity that a

FIGURE 1: Specificity of Binding of Fully-Modified Methylphosphonate Oligodeoxynucleotide 12-Me[10] to Complementary DNA.



Panel **a**: Ethidium bromide stained 1% agarose gel. Lane 1 = EcoRI, HindIII, and RsaI digested plasmid DNA containing the complementary sequence. Lane 2 = HindIII digested Lambda DNA. Lane 3 = HaeIII digested X174 DNA.

Panel **b**: Autoradiograph of Southern hybridization of the same gel.

methylphosphonate oligodeoxynucleotide has for its complementary sequence versus unrelated sequences, Southern (1975) hybridization was performed in 1 M sodium ion on restriction endonuclease digested pALA–D, as well as control DNAs, using 5' ^{32}P–labeled, oligodeoxynucleotide 12–Me^{10}. This is the fully substituted oligodeoxynucleotide, having the most diastereoisomeric forms of all of the methylphosphonate oligodeoxynucleotides in this study. Hybridization was carried out at 37°C for 30 minutes and continued for 2 hours while the temperature decreased to room temperature. This temperature range was chosen because the dissociation temperature had previously been determined to be between 30 and 35°C (see dot blots below).

Figure 1a shows the EcoRI/HindIII/RsaI restriction enzyme digest of pALA–D DNA separated on a 1% agarose gel. The indicated (796 bp) fragment contained the sequence complementary to oligodeoxynucleotide 12–Me^{10}. Figure 1b shows the autoradiogram of the Southern hybridization demonstrating that ^{32}P–labeled 12–Me^{10} bound specifically to the 796 bp fragment of pALA–D. In addition, no binding of this oligodeoxynucleotide was observed to either of the digested phage DNAs. A search of the bacteriophage lambda DNA sequence revealed four regions of complementarity with only two mismatches, in one case with an uninterrupted homology of 10 nucleotide pairs. Thus, a fully–substituted oligodeoxynucleotide maintains a high degree of specificity for its complementary DNA sequence.

2. CHARACTERISTIC TEMPERATURES AND THE THERMODYNAMICS OF DNA MELTING

There are three temperatures characteristic of oligodeoxynucleotide binding to complementary DNA:

T_m^{∞}: The DNA melting temperature is defined to be the temperature at which an infinitely long DNA molecule is half denatured. T_m^{∞} depends on DNA base composition and on the properties of the solvent, including the ionic strength.

T_m: The oligodeoxynucleotide melting temperature is defined to be the temperature at which 50 percent of the nucleotides of an equimolar mixture of complementary oligodeoxynucleotides are in the duplex state. T_m depends upon oligodeoxynucleotide concentration in addition to the nucleotide sequence and on the properties of the solvent.

T_d: The dissociation temperature ($T_d = t_d + 273.16$) is defined as the temperature at which 50% of labeled oligodeoxynucleotide is dissociated from complementary DNA after a specified time. T_d depends on time and the nucleotide sequence and the properties of the solvent but is independent of concentration. T_d is related to a kinetic and not a thermodynamic property of the duplex. The specified time for 50% dissociation (half–time for the reaction) is equal to ln 2 divided by the rate constant for a helix to coil transition at T_d.

Breslauer, et al. (1986), using the methods in Marky and Breslauer (1987), have analyzed the melting of DNA and complementary oligodeoxynucleotides in terms of nearest neighbor contributions to the enthalpy of base pair formation. For a long homo– or heteropolymeric DNA, the helix–coil transition enthalpy thus determined, $\Delta H°_{av}$, is the average of the contributions of the

various nearest neighbor enthalpies. Letting T° = 298.16°K be the reference temperature, the average nearest–neighbor free energy of the helix–coil transition is given by:

$$\Delta G°_{av} = \Delta H°_{av} (1 - T°/T_m^{\infty}) \qquad \text{(eq 1)}$$

A similar analysis was carried out for two–state helix–coil transitions involving complementary oligodeoxynucleotides by assuming that no enthalpy is associated with the formation of the first base pair. Then all thermodynamic parameters may still be associated with nearest neighbor interactions. Defining ΔG° and a temperature–independent ΔH° to be the standard free energy and enthalpy for the sum of all nearest neighbor contributions to the helix–coil transition and letting C be the total concentration of both of the complementary oligodeoxynucleotides,

$$\Delta G° = \Delta H° (1 - T°/T_m) - RT° \ln(C/4) \qquad \text{(eq 2)}$$

As the molecules become large, the last term becomes increasingly insignificant. ΔH° becomes very large and T_m approaches T_m^{∞}. For self–complementary oligodeoxynucleotides, the RT° ln(C/4) term becomes RT° ln(C/2).

3. CALCULATION OF NEAREST NEIGHBOR FREE ENERGIES

Breslauer, et al. (1986) stated that their results failed to predict T_m values correctly for oligodeoxynucleotides containing GG nearest neighbors. Because one of the oligodeoxynucleotides involved in the study of the effect of methylphosphonate linkages contained three adjacent GG interactions, we decided to reexamine the thermodynamic free energy parameters involved in helix–coil transitions to see if such molecules were indeed exceptions. All

enthalpy parameters used were those determined by Breslauer, et al. (1986).

The basis set used for determining nearest–neighbor free energy parameters is given in Table 2 together with references to the sources of the data. The basis set included approximately equal weighting for oligo–deoxynucleotides, including several with adjacent GG nearest neighbors, heteropolymers, and natural DNAs of various base compositions. Finally, a penalty was imposed for each nearest neighbor interaction for deviation from simple dependence on base composition. These data were entered into the MGLH program of SYSTAT, Inc. (Evanston, IL) for calculation of nearest neighbor free energies and the intercept, $\Delta G°_i$, the negative of the free energy for helix initiation, which best fit all of the data for both oligodeoxynucleotide duplex formation and melting of long DNAs as described by equations (1) and (2) above. The symmetry term for self–complementary oligodeoxynucleotides was treated in the same manner as Breslauer, et al. (1986). $\Delta G°_{nn}$, the nearest–neighbor free energies are listed at the top of Table 2. Standard Errors for all $\Delta G°_{nn}$ were approximately 0.04. $\Delta G°_i$ was determined to be –2.2 kcal/mol (–2.6 kcal/mol if self–complementary). Identical values have been determined for polymers containing dG and dC nucleotides (Pohl, 1974). Unlike the calculations of Breslauer, et al. (1986), no assumption about the length of the cooperative unit for melting of large DNAs was built into the model. The resulting low calculated contribution of the initiation term to the free energies for oligodeoxynucleotide helix–coil transitions may derive from a dependence of the enthalpy on temperature.

The standard error for calculated and experimental $\Delta G°$ is 7%. The calculated and experimental T_m values (DNAs) differ by no more than 1°C. The $\Delta G°$ values for oligodeoxynucleotides of known concentration are easily

TABLE 2

	AA TT	AT AT	TA TA	CA TG	GT AC	CT AG	GA TC	CG CG	GC GC	GG CC	Symm. 1 or 0	Delta H° Calc	Delta G° Expt	Delta G° Calc	Tm Expt	Tm Calc	Conc. in uM	Reference
NEAREST NEIGHBOR VALUES:																		
ENTHALPY:	9.10	8.60	6.00	5.80	6.50	7.80	5.60	11.90	11.10	11.00								
FREE ENERGY:	1.55	1.25	0.85	1.15	1.40	1.45	1.15	3.05	2.70	2.30								
ENTROPY (cal):	25.32	24.65	17.27	15.60	17.10	21.30	14.92	29.68	26.17	29.18								
INITIATION FREE ENERGY:	2.2		SYMMETRY FACTOR:		0.4													
NEAREST NEIGHBORS:																		
OLIGODEOXYRIBONUCLEOTIDES:																		
GCGCGC	0	0	0	0	0	0	0	2	3	0	1	57.1	11.1	11.60				1
CGTCGACG	0	0	0	0	2	0	2	3	0	0	1	59.9	11.9	11.65				1
GAAGCTTC	2	0	0	0	0	2	2	0	1	0	1	56.1	8.7	8.40				1
GCAATTCC	2	1	0	0	0	0	2	0	0	2	1	60	9.4	8.65	36	36	18.8	1 and 2
GGTATACC	0	1	2	0	2	0	0	0	0	2	1	55.6	7.4	7.75				1
GCGAATTCGC	2	1	0	0	0	0	2	2	2	0	1	64	15.5	15.55				1
CGCGAATTCGCG	2	1	0	0	0	0	2	4	2	0	1	107.8		21.65	73	77	88.6	3
CGCGTACGCGTACGCG	0	0	2	0	4	0	0	6	3	0	1	142.7	34.1	31.10				1
CCCGGG	0	0	0	0	0	0	0	1	0	4	1	55.9		9.65	48	49	100	4
CCCAGGG	0	0	0	1	0	1	0	0	0	4	0	57.6		9.60	43	43	100	4
GATGGGCAG	0	1	0	2	0	1	1	0	1	2	0	66.7		11.25	46	49	100	5
CAAAAAG	4	0	0	1	0	1	0	0	0	0	0	50	6.1	6.60				1
CAAACAAAG	4	0	0	2	1	1	0	0	0	0	0	62.3	10.1	9.15				1
CAAAAAAAG	6	0	0	1	0	1	0	0	0	0	0	68.2	9.6	9.70				1
CAAATAAAG	4	1	1	1	0	1	0	0	0	0	0	64.6	8.5	8.70				1
CAAAGAAAG	4	0	0	1	0	2	1	0	0	0	0	63.4	9.5	9.20				1
TGGGGCTGCATG	0	1	0	4	0	1	0	0	2	3	0	94.8		17.40	60	58	7	This work
TGACTTAGCTGCAT	1	1	1	3	1	3	1	0	2	0	0	98.8		17.20	57	55	6	This work
POLYMERS:																		
d(GC)-d(GC)	0	0	0	0	0	0	0	10	10	0	0	230		57.50	123	124		6
d(AC)-d(GT)	0	0	0	10	10	0	0	0	0	0	0	123		25.50	101	103		6
d(GAT)-d(ATC)	0	7	0	7	0	0	7	0	0	0	0	140		24.85	98	89		6
d(AAC)-d(GTT)	7	0	0	7	7	0	0	0	0	0	0	149.8		28.70	97	98		6
d(AG)-d(CT)	0	0	0	0	0	10	10	0	0	0	0	134		26.00	98	97		6
d(AGT)-d(ACT)	0	0	7	0	7	7	0	0	0	0	0	142.1		25.90	93	91		6
d(TTC)-d(GAA)	7	0	0	0	0	7	7	0	0	0	0	157.5		29.05	94	92		6
d(AAT)-d(ATT)	7	7	7	0	0	0	0	0	0	0	0	165.9		25.55	79	79		7
d(AT)-d(AT)	0	10	10	0	0	0	0	0	0	0	0	148		21.00	75	75		6
NATURAL DNAs:																		
20% GC	6	4	4	2	2	2	2	0.25	0.25	0.5	0	193.85		33.69	87	88		8
40% GC	4.5	2.25	2.25	3	3	3	3	1	1	2	0	195.9		37.50	95	96		8
60% GC	2	1	1	3	3	3	3	2.25	2.25	4.5	0	211.15		43.94	102	103		8
80% GC	0.5	0.25	0.25	2	2	2	2	4	4	6	0	239.6		53.00	110	110		8
NEAREST NEIGHBOR INTERACTIONS:																		
AT	0	20	0	0	0	0	0	0	0	0	0	172		25.00	75	76		-
TA	0	0	20	0	0	0	0	0	0	0	0	120		17.00	75	74		-
CA	0	0	0	20	0	0	0	0	0	0	0	116		23.00	101	99		-
GT	0	0	0	0	20	0	0	0	0	0	0	130		28.00	101	107		-
CT	0	0	0	0	0	20	0	0	0	0	0	156		29.00	96	93		-
GA	0	0	0	0	0	0	20	0	0	0	0	112		23.00	96	102		-
CG	0	0	0	0	0	0	0	20	0	0	0	230		61.00	123	128		-
GC	0	0	0	0	0	0	0	0	20	0	0	222		54.00	123	121		-
POLYMERS NOT IN THE BASIS SET:																		
d(A)	20	0	0	0	0	0	0	0	0	0	0	182		31.00	86	86		6
d(G)	0	0	0	0	0	0	0	0	0	20	0	220		46.00		104		-
NUMBER OF TIMES IN BASIS SET - SEE TEXT:	258	145	137	192	168	184	180	157	149	158								

CAPTION FOR TABLE 2

ENTHALPY ($\Delta H°$) DATA ARE FROM Breslauer, et al. (1986).

EXPERIMENTAL DATA (all converted into $\Delta G°$ values):

A. Sources:

1. Breslauer, et al. (1986).
2. Bower, et al. (1987).
3. Marky, et al. (1983).
4. Arnold, et al. (1987).
5. Woodson & Crothers (1987).
6. Wells, et al. (1970). (Corrected for length effects)
7. Ornstein & Fresco (1983).
8. Marmur & Doty (1962). (Corrected to 1 M NaCl)
9. This work.

B. Weighting (To equalize contributions of nucleotides)

Oligodeoxynucleotides: 4

Polymers: 2 Nearest–neighbor interactions: 1

Natural DNAs: 6

converted into T_m values by using a rearranged form of equation 2. The standard error for calculated and experimental T_m values is 2°C. Thus, a single formulation fits well all types of DNA melting analyses, including helix–coil transitions of oligodeoxynucleotides containing adjacent GG nearest neighbors.

The calculated T_m^{∞} for poly d(AT) d(AT), taken as an unknown, agreed with experiment. The calculated t_m^{∞} for poly dG dC was 104°C in 1 M NaCl. This value is lower than experimental values extrapolated to 1 M NaCl. Poly dG dC thermodynamic data may be anomalous, and its inclusion in the data used for interpreting other DNA hybridization experiments may be erroneous.

4. THE EFFECT OF DANGLING ENDS

Both oligodeoxynucleotides used for this study were hybridized to recipient molecules overlapping at both the 5' and 3' ends. The contribution of dangling ends was found to be about 1 kcal/mol (Table 3), leading to an increase in T_m of 3–4°C. The 14 + 18–mer duplex with 5' and 3' adjacent dangling purines was slightly (1°C) more stable than the duplex with 5' and 3' adjacent dangling purines. The magnitude of the result is in line with other studies of dangling ends (Senior, et al., 1988).

TABLE 3: Melting Temperatures (t_m) for Phosphodiester and Methylphosphonate–Containing Oligodeoxynucleotides

EFFECT OF DANGLING ENDS IN 1.0 M NaCl:

OLIGODEOXYNUCLEOTIDES*:	t_m (°C)
12–P1 + 12–P2	60°
12–P1 + 18–P3	63°
14–P1 + 14–P2	57°
14–P1 + 18–P1	61°
14–P2 + 18–P2	60°

EFFECT OF IONIC STRENGTH:

	SALT CONCENTRATION			
OLIGODEOXYNUCLEOTIDE*	1.0	0.1	0.02	0.004
Unmodified:				
14–P1	61°	53°	42°	31°
14–P2	60°			
14–P Calculated	60°	52°	42°	31°
Partially substituted (3 methylphosphonates on one strand):				
14–A	52°	41°	36°	30°
14–C	50°	45°	38°	32°
14–G	53°	42°	—	32°
14–T	52°	43°	39°	30°
14–Me[3] Calculated	52°	46°	38°	30°

*(Total strand concentration = 6 μM)

5. THE EFFECT OF IONIC STRENGTH ON THE T_m FOR PHOSPHODIESTER OR PARTIALLY METHYLPHOSPHONATE SUBSTITUTED OLIGODEOXYNUCLEOTIDES BOUND TO COMPLEMENTARY OLIGODEOXYNUCLEOTIDES WITH 5' AND 3' DANGLING ENDS

Table 3 lists melting temperatures determined for phosphodiester and partially methylphosphonate–substituted oligodeoxynucleotides at various ionic strengths. At 100 mM NaCl methylphosphonate and phosphodiester oligodeoxynucleotides have equivalent hyperchromicities, indicating that all of the diastereoisomers for each of the methylphosphonate–substituted oligodeoxynucleotides form duplexes with their respective recipients. The width of the helix–coil transition for 14–A + 18–P1 was 3°C greater than that for 14–P1 + 18–P1. This difference can be explained by the 2.5°C difference in T_m values for the R and S diastereoisomers at each chiral center (Bower, et al., 1987).

The t_m values reported in Table 3 include those for 18–P1 plus either 14–P1, 14–A or 14–C as well as 18–P2 plus either 14–P2, 14–G or 14–C. The oligodeoxynucleotides 14–A, –C, –G, and –T contained three non–adjacent methylphosphonate linkages 3' to the A, C, G or T nucleotides. In 1 M NaCl, substitution of these methylphosphonate linkages resulted in a decrease in t_m of 7–11°C. There does not appear to be a large sequence specificity for the destabilization due to nonadjacent methylphosphonate bonds.

The free energy of destabilization associated with one such bond is defined as $\Delta G°_d$. An additional free energy term may be introduced to account for the effect of decreasing ionic strength on T_m, $\Delta G°_s$. $\Delta G°_s$ is taken to be 0

in 1 M NaCl. $\Delta G°_e$, the dangling ends term, is taken to be +1 kcal/mol, and $\Delta G°_i$, the initiation term, is taken to be –2.2 kcal/mol. Calculated T_m values in Table 3 were increased by 2°C to correct for the difference between experimental and theoretical results for 14–P1 + 14–P2 shown in Table 2.

In summary, calculated melting temperatures were obtained using:

$$T_m = T° \Delta H° / (\Delta H° - \Delta G° - R\ T° \ln [C/4]) \qquad \text{(Eq 3)}$$

where $\Delta H° = \Sigma_{nn} (N_{nn}\ \Delta H°_{nn})$

and $\Delta G° = \Sigma_{nn} (N_{nn}\ \Delta G°_{nn}) + \Delta G°_i + \Delta G°_e + N_{Me}\ \Delta G°_d + \Delta G°_s$

$\Delta G°_d$ was found to be –0.75 kcal/mol, leading to a substantial decrease in T_m for methylphosphonate–substituted oligodeoxynucleotides at 1 M NaCl. This result is compatible with those of Bower, et al. (1987). The ionic strength data were fit to:

$$\Delta G°_s = (n_h - n_c)\ R\ T° \{\ln[M/(0.3+M)] + \ln[1.3]\} \qquad \text{(Eq 4)}$$

where $(n_h - n_c)$ is the change in total charge difference between the helix and coil forms, and M is the sodium ion concentration.

For each nearest neighbor base pair in phosphodiester DNA:

$$(n_h - n_c)\ /(\Sigma_{nn}\ N_{nn}) = 0.26$$

This value is greater than predicted by Record and Lohman (1978) and indicates the need for more theoretical work aimed toward explaining the association of counterions with oligodeoxynucleotides.

For each nearest neighbor base pair in methylphosphonate DNA:

$$(n_h - n_c) = 0$$

The calculated t_m values using Eq 3 and these $(n_h - n_c)$ results are compared with experimental t_m values obtained at various ionic strengths in Table 3. The fit between experimental and theoretical t_m values is quite satisfactory.

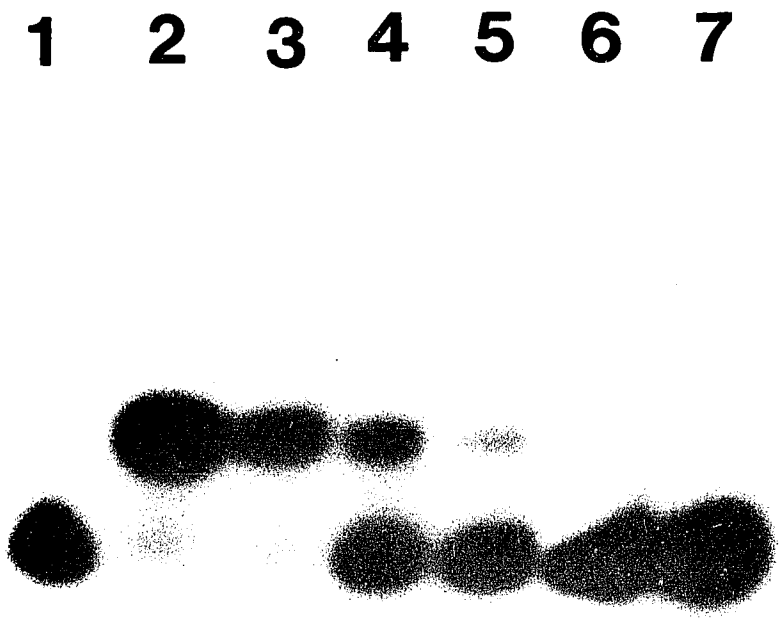
In low salt, a higher relative stability for methylphosphonate–containing

oligodeoxynucleotides is seen where T_m values for either partially (Table 3) or fully (Figure 4, below) methylphosphonate–substituted oligodeoxynucleotides become greater than those of the analogous phosphodiesters either at or below 4mM NaCl.

6. DETERMINATION OF BINDING OF 12–Me^{10} TO 18–P3 USING THE POLYACRYLAMIDE GEL MIGRATION ASSAY

As demonstrated in Figure 2, duplex formation between a phosphodiester oligodeoxynucleotide that has been 5'–^{32}P–labeled (18–P3) and a complementary oligodeoxynucleotide (12–Me^{10}) can be demonstrated by the altered gel mobility of the band containing the labeled oligodeoxynucleotide. In addition, the stoichiometry of hybridization may be quantitated by such an analysis. It is often difficult to determine precise concentrations (μg/ml) of fully–substituted methylphosphonate oligodeoxynucleotides by ultraviolet spectrophotometry. More importantly, no optical melting curve can be obtained for such a large mixture of chiral forms of differing duplex stabilities. With the gel migration assay, a set quantity of recipient oligodeoxynucleotide may be titrated with varying amounts of methylphosphonate–substituted complementary oligodeoxynucleotide to determine the arbitrary absorbance ratio which allows complete hybridization. Figure 2 shows that a ratio of 1:10 for 18–P3:12–Me^{10} is sufficient for complete hybridization, whereas 1:5 is not sufficient. All hybridization mixtures of 18–P3 and 12–Me^{10} for subsequent gel migration studies were prepared using the 1:10 ratio. Using the gel migration assay, the migration distances of duplexes formed between a recipient and either complementary phosphodiester or methylphosphonate

FIGURE 2: Determination of the Stoichiometry of Binding of Fully–Modified Methylphosphonate Oligodeoxynucleotide 12–Me^{10} to ^{32}P–Labeled Complementary 18–P3.



Complementary oligodeoxynucleotides were annealed in 100 mM salt at room temperature. Electrophoresis was carried out in a 20% polyacrylamide gel at 4°C in TBE. Lane 1: 18–P3 only. Lanes 2–7: 18–P3 + 12–Me^{10} at 1:20, 1:10, 1:5, 1:2.5, 1:1.25, 1:0.625 (arbitrary units).

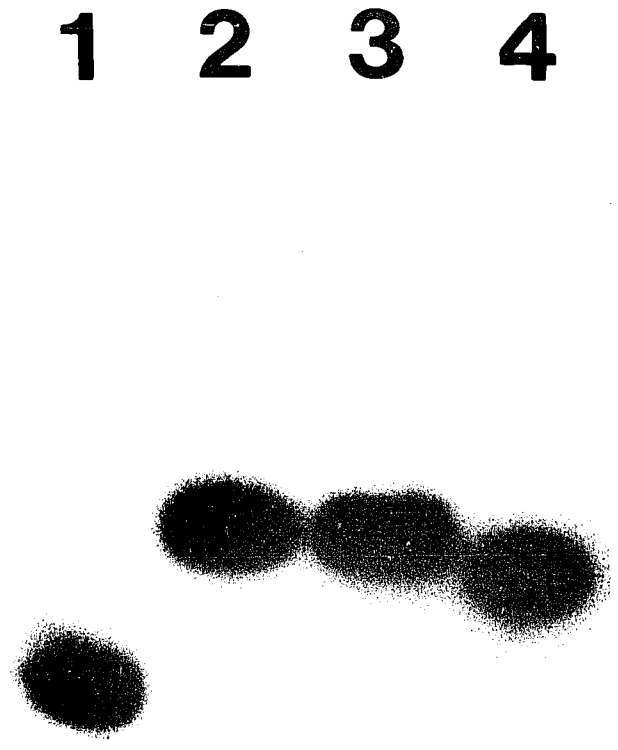
sequences may be compared. Figure 3 shows a distinct difference between the migration for the 18–P3:12–Me^{10} (lane 2) and 18–P3:12–P1 (lane 4) duplexes. The duplex containing the reduced–charge oligodeoxynucleotide has a lower mobility than the duplex containing the equivalent phosphodiester oligodeoxynucleotide.

The gel migration assay was also used to determine the extent to which duplexes with labeled recipient strands, once formed, would exchange complementary strands. As demonstrated in lane 3, Figure 3, the addition of 12–P1 to the annealed 18–P3:12–Me^{10} hybrid does not displace oligodeoxynucleotide 12–Me^{10} (100mM NaCl, TE/5 at 4°C). Thus the gel migration assay may be used to quantitate the products of hybridization reactions carried out at elevated temperatures in various salt solutions.

Finally, the gel migration assay was used to determine the ionic strength dependence of the preference for binding of 12–P1 versus 12–Me^{10} to 18–P3. Mixtures were made containing a 1:10 ratio of 18–P3:12–Me^{10} and a 1:1 ratio of 18–P3:12–P1 at various salt concentrations: TE/5, 10mM NaCl in TE/5, 100mM NaCl in TE/5, and 1 M NaCl in TE/5. After a 5 minute incubation at 70°C to assure complete denaturation of any transiently formed duplexes, the mixture was slowly cooled to room temperature, cooled to 4°C and assayed by gel electrophoresis. The results in Figure 4 show that 18–P3 hybridizes preferentially to 12–P1 in the 1 M and 100mM NaCl conditions, is divided between 12–P1 and 12–Me^{10} in 10mM NaCl, and binds preferentially to 12–Me^{10} in TE/5. Thus, at low salt, the fully–substituted methylphosphonate containing oligodeoxynucleotide forms more stable hybrids than analogous phosphodiester sequences.

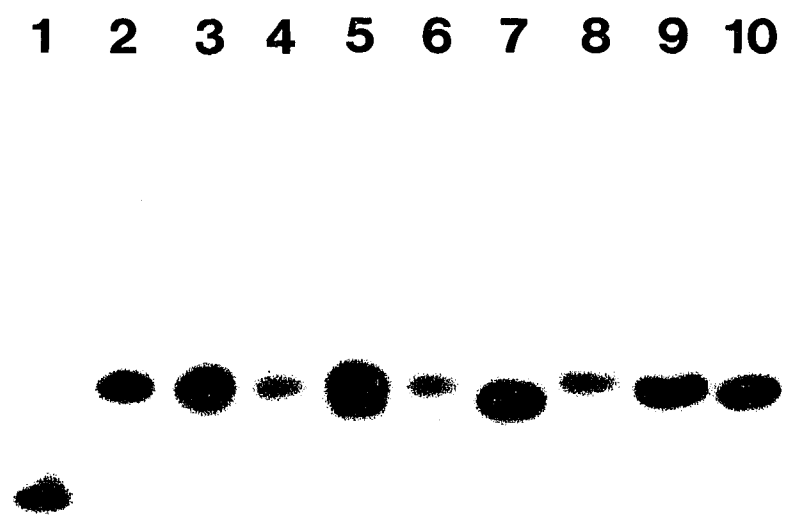
Using Eq 3 and 4, predicted t_m values for 12–Me^{10} range from 34°C in

FIGURE 3: Fully–Modified Methylphosphonate Oligodeoxynucleotide 12–Me^{10} Bound to ^{32}P–Labeled Complementary 18–P3 is not Displaced at 4°C.



Complementary oligodeoxynucleotides were annealed in 100 mM NaCl at room temperature. Electrophoresis was performed in a 20% polyacrylamide gel at 4°C in TBE. Lane 1: 18–P3 only. Lane 2: 18–P3 + 12–Me^{10}. Lane 3: 18–P3 + 12–Me^{10} followed by 12–P1. Lane 4: 18–P3 + 12–P1.

FIGURE 4: Fully–Modified Methylphosphonate Oligodeoxynucleotide 12–Me^{10} and Unmodified Oligodeoxynucleotide 12–P1 Compete for ^{32}P–Labeled Complementary Oligodeoxynucleotide 18–P3.



Oligodeoxynucleotides were heated to 70°C and slow–cooled to room temperature at various ionic strengths. Electrophoresis was performed as described in Fig. 3. Competing oligodeoxynucleotide and sodium ion concentration were: Lane 1: None, 0.1. Lane 2: 12–Me^{10}, 0.002. Lane 3: 12–P1, 0.002. Lane 4: 12–Me^{10}, 0.01. Lane 5: 12–P1, 0.01. Lane 6: 12–Me^{10}, 0.1. Lane 7: 12–P1, 0.1. Lane 8: 12–Me^{10}, 1.0. Lane 9: 12–P1, 1.0. Lane 10: 12–P1, 0.1.

1 M NaCl to 31°C in 0.002 M NaCl. These results predict that t_m for 18–P3:12–Me^{10} and 18–P3:12–P1 hybrids would not become identical until the salt concentration was reduced to 0.002 M, whereas the results in Figure 4 indicate equivalent t_m values in 0.01 M NaCl plus TE/5. One explanation for the large apparent relative affinity of 12–Me^{10} for 18–P3 is that the chiral mixture which constitutes 12–Me^{10} contains many diastereoisomers with smaller detrimental $\Delta G°_d$ values than the –0.75 kcal/mol average value used for the calculations. A corollary to this explanation is that only a subset of the diastereoisomers present in fully methylphosphonate–substituted oligodeoxynucleotides may be capable of participating in duplex formation.

7. THE EFFECT OF IONIC STRENGTH ON THE DISSOCIATION TEMPERATURE OF NORMAL AND BACKBONE–MODIFIED OLIGODEOXYNUCLEOTIDES BOUND TO COMPLEMENTARY DNA

Dot–blot filters were prepared by spotting up to 200 fM of denatured pALA–D DNA per dot. Duplexes were formed by incubation of the filters with 100–fold excess ^{32}P–labeled oligodeoxynucleotides at 37°C for 30 minutes, cooling to room temperature and incubation for an additional 90 minutes. Based on the specific activity of the oligodeoxynucleotides, no more than 20 fM of bound oligodeoxynucleotide could be released from any filter segment containing a dot. That is, at most 10% of the sites on pALA–D contained bound oligodeoxynucleotide. Table 4 shows the temperature of 50% dissociation (t_d) of bound oligodeoxynucleotides. Each temperature point of a dissociation curve was determined using an identical dot which was washed for 30 minutes into 10 ml or more of the solvent indicated. Either intermittent

TABLE 4: Dissociation Temperatures (t_d) for Methylphosphonate–Containing Oligodeoxynucleotides

t_d in 1 M SODIUM ION:

	OLIGODEOXYNUCLEOTIDE	t_d (°C)
Unmodified:	14–P1	41°
Partially modified:	14–A	32°
Fully modified:	12–Me[10]	30–35°

t_d in 0.01 M SODIUM ION:

	OLIGODEOXYNUCLEOTIDE	t_d
Unmodified:	14–P1	33°
Partially modified:	14–A	32°
	14–C	30°
	14–G	25–30°
	14–T	30°
Fully modified:	12–Me[10]	35–40°

t_d: THE TEMPERATURE AT WHICH 50% OF LABELED OLIGODEOXY-NUCLEOTIDE IS DISSOCIATED FROM COMPLEMENTARY DNA AFTER 30 MINUTES OF WASHING

manual or continuous mechanical stirring was employed during the washing procedure. In no case did the results depend on the method of stirring, although no attempt was made to measure t_d with restricted mixing. Some preliminary experiments had been carried out, with identical results, using the same protocol and manually synthesized (Miller, et al., 1986) methylphosphonate oligodeoxynucleotides.

t_d is the temperature of importance for screening plasmid or bacteriophage recombinant DNA libraries using oligodeoxynucleotide probes. Suggs, et al. (1981) proposed an empirical rule of thumb for calculating t_d in 1 M NaCl: the sum of 2°C for each dA dT base pair and 4°C for each dG dC base pair. Using this rule, t_d for 14–P1 should be 40°C. The observed value of 41°C is in good agreement with the rule. Based on the model proposed above, the specified time for 50% dissociation at T_d is equal to ln2 divided by the reverse rate constant for duplex formation, k_r. Thus, k_r is ln2 / 1800 at T_d when the specified time is 30 minutes.

Using the definition of k_2 for DNA duplex formation from Wetmur and Davidson (1968) and defining C_o to be the total nucleotide concentration of non–self–complementary oligodeoxynucleotides, k_2 / k_r is equal to 4 / C_o at T_m. k_2 may thus be determined at T_m if k_r, which is known at T_d, can be determined at T_m by extrapolation using E_r^*, the activation energy for k_r. If E_2^* is the activation energy for k_2, then:

$$\Delta H^\circ = E_r^* - E_2^* \qquad \text{(Eq 5)}$$

E_2^* has been determined for both DNA and oligonucleotide reassociation rates to be very small compared to E_r^* (Reviewed by Wetmur, 1976). An appropriate estimate for E_2^* would be 4 kcal/mol, approximately that which could be accounted for by a diffusion–controlled rate determining step

depending on T / microscopic solvent viscosity. Thus, for 14–P1 (or 14–A) in 1 M salt, E_r^* would be approximately 103 kcal/mol. The relation between T_d and T_m and the reassociation rate constant k_2 is:

$$k_2 = \frac{4}{C_o} \frac{\ln 2}{1800} \text{EXP} \{ \frac{E_r^*}{R} [\frac{1}{T_d} - \frac{1}{T_m}] \} \qquad \text{(Eq 6)}$$

The nucleation rate constant for DNA reassociation, k_N', is given by:

$k_N' = k_2 \quad N / L^{0.5}$, where N is complexity and L is strand length.

For non–self–complementary oligodeoxynucleotides, L and N are the same. Wetmur and Davidson (1968) found k_N' for DNA in this same solvent to be 5 x 10^5 $M^{-1}sec^{-1}$ after applying a 3/2 correction to obtain a value for nonpermuted molecules. Lee and Wetmur (1972) observed that k_N' was approximately the same for oligonucleotides and polymers.

During washing at t_d, the half–time for rebinding free oligodeoxy–nucleotide would be ln2 / k_2 C_o where C_o for available target pALA–D DNA (10–200 fM in 10 ml) would be 0.14–2.8 x 10^{-10} M nucleotide. k_2 = 3.5 x 10^5 / $14^{0.5}$ at T_d. Thus, the half–time for rebinding could be as short as 7.35 hours. However, because the dots were initially saturated with oligodeoxynucleotide, a better estimate of the half–time for rebinding would be in excess of 100 hours. In either case, because the rebinding rate is so low, t_d is a measure of k_r and, unlike t_m, is not an equilibrium property. Furthermore, because k_2 decreases dramatically when the ionic strength is decreased, all measurements in salts below 1 M Na^+ would involve even longer rebinding half–times. Clearly, if the concentration of oligodeoxynucleotide were higher during washing, t_d could become a measure of an equilibrium property. When t_d is a kinetic measurement, increasing the washing time, but not the volume, will lead to decreased retention of radioactivity on a dot. If t_d is an equilibrium

measurement, increasing the volume, but not the washing time, will lead to decreased retention of radioactivity on a dot.

From Table 3, t_m for 14–P1 is 61°C and t_m for 14–A is 52°C when C_o is 84 μM. Using a rearranged form of Eq 6, t_d for 14–P1 is calculated to be 43°C and t_d for 14–A is calculated to be 34°C. In both cases, the predicted t_d is 2–3°C higher than the experimental value. This small difference could be the result of:

1) a temperature dependence of $\Delta H°$ leading to a smaller E_r^*,
2) a lower t_d for ^{32}P–labeled DNA than unlabeled DNA used to determine t_m,
3) incomplete equivalence of the dangling ends for the t_m measurement with the extended strands involved in the t_d measurement, or any combination of these factors.

The agreement between the results with the phosphodiester 14–P1 and the methylphosphonate–substituted 14–A is remarkable because the apparent k_r for 14–A reflects elution of the various chiral forms (Bower, et al., 1987) of 14–A and is not a true rate constant. Nevertheless, we may conclude that the difference in T_m between phosphodiester and partially methylphosphonate–substituted oligodeoxynucleotides reflects primarily a difference in the reverse rate constant k_r.

For 14–P1, T_m decreases 24°C while T_d decreases 8°C going from 1.0 M NaCl to 0.01 M NaCl, indicating that the major effect of changing the ionic strength is on the forward rate constant. Porschke, Uhlenbeck and Martin (1973) found that A_NGCU_N duplex strand separation rates were the same at all temperatures in both 0.05 M NaCl and 1.0 M NaCl for both N=2 and N=4, indicating either a small or no dependence of T_d on ionic strength for these oligoribonucleotides. In the case of partially or fully methylphosphonate

substituted oligodeoxynucleotides, no ionic strength effect is seen on T_d. In addition, T_d is not very dependent on the nature of the modified base. Interpretation of these results in terms of rate constants is somewhat complicated by the mixture of chiral forms with different equilibrium constants. The precise rate constants could only be obtained using a combination of stopped–flow and temperature–jump measurements.

III.

THE EFFECT OF THE NUMBER AND DISTRIBUTION OF METHYLPHOSPHONATE LINKAGES IN OLIGODEOXYNUCLEOTIDES ON SENSITIVITY TO EXO- AND ENDONUCLEASES AND ON ABILITY TO FORM RNASE H SUBSTRATES

A. INTRODUCTION

Oligodeoxynucleotides are of interest today as antisense agents to inhibit gene expression by hybrid arrest of translation. One method of achieving a high degree of efficiency in translation inhibition is to chemically alter the oligodeoxynucleotide in order to facilitate its entry into the cell and to increase its half-life, while maintaining its affinity for the specific mRNA. Oligodeoxynucleotide analogues containing methylphosphonate linkages have been shown to have an antisense effect in various in vitro (Miller, et al., 1985) and tissue culture (Smith, et al., 1986; Agris, et al., 1986; Sarin, et al., 1988) systems. The non-ionic linkages appear to enhance entry into cells and the altered chemical structure renders such compounds resistant to various nucleases leading to a long half life in culture medium and within cells (Miller, et al., 1981).

One mechanism by which phosphodiester oligodeoxynucleotides have been found to promote hybrid arrest of translation is through RNase H cleavage of the RNA in the RNA:oligodeoxynucleotide duplex (Minshull & Hunt, 1986; Cazenave, et al., 1987; Dash, et al., 1987). In a cell-free translation system, it was determined that the action of RNase H was not involved in the antisense effect seen with fully methylphosphonate-substituted oligodeoxynucleotides, and that such compounds did not serve as RNase H substrates when hybridized to complementary RNA (Maher & Dolnick, 1988).

In this paper we examine the effect of the number and positioning of methylphosphonate substitutions in oligodeoxynucleotides on nuclease sensitivity, stability in tissue culture, and ability to form RNase H substrates.

B. MATERIALS AND METHODS

1. OLIGODEOXYNCULEOTIDES AND PLASMIDS

All oligodeoxynucleotides (Table 5) were synthesized on an Applied Biosystems Model 380B DNA Synthesizer. Phosphodiester linkages were generated by standard phosphoramidite chemistry, and methylphosphonate bonds were introduced by the coupling of methylphosphonamidite monomers. Hydrolysis of base–protecting groups and cleavage from the support for phosphodiester oligodeoxynucleotides was accomplished by NH_4OH treatment, which was followed by ethanol precipitation. Oligodeoxynucleotides containing mixed phosphodiester and methylphosphonate bonds were released and deprotected in ethylenediamine:ethanol (1:1) for 7 hours at room temperature (Miller, et al., 1986). To increase the yield, the support material was also treated in NH_4OH for 2 hours at room temperature (Agrawal, et al., 1988). These oligodeoxynucleotides were purified by 4M ammonium acetate elution from NACS Prepac columns (Bethesda Research Labs). All oligodeoxynucleotides were 5'–labeled with ^{32}P using T4 polynucleotide kinase, subjected to polyacrylamide gel electrophoresis on 20% acrylamide–8M urea gels, and visualized by autoradiography. All methylphosphonate–substituted oligodeoxynucleotides were quantitated by the ability to induce an electrophoretic mobility shift of their respective 5'–^{32}P–labeled complementary phosphodiester oligodeoxynucleotides (Chapter II; Quartin & Wetmur, 1989). pSP65–ALA–D is a derivative of the SP6 cloning vector pSP65 (Melton, et al., 1984) containing the cDNA sequence of human delta–aminolevulinic acid dehydratase (ALA–D), a heme biosynthetic enzyme (Wetmur, et al., 1986),

inserted in the sense orientation in the PstI site of the polylinker.

2. EXO– AND ENDONUCLEASE DIGESTS

Nuclease digestions of oligodeoxynucleotides were carried out in 10 µl volumes under the conditions described below for each enzyme. Enzyme quantities for digestion reactions and half–times for digestion were determined by titration with phosphodiester oligodeoxynucleotide controls:

(1) One µg bovine pancreas DNase I (3.1.4.5) (Bethesda Research Laboratories): 50 mM sodium acetate (pH 6.5), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 37°C.

(2) Twenty units bovine spleen DNase II (3.1.4.6) (Bethesda Research Laboratories): 0.8 mM $MgSO_4$, 83.3 mM HOAc (pH 4.6), 25°C.

(3) Thirty mU Bovine spleen phosphodiesterase (3.1.4.18) (Sigma): 0.1 M sodium citrate (pH 6.0), 5 mM EDTA, 37°C.

(4) Ten µU snake venom phosphodiesterase from Crotalus adamanteus (3.1.4.1) (Sigma): 0.2 mM Tris–Cl (pH 8.9), 37°C.

DNase I and snake venom phosphodiesterase reactions were stopped by addition of EDTA to 25 mM and incubation at 70°C for 10 minutes. Spleen phosphodiesterase reactions were stopped by incubation at 70°C for 10 minutes. DNase II was inactivated by addition of EDTA to 21 mM and Tris–OH to pH 6.0, followed by incubation at 70°C for 30 minutes. Annealing reactions were carried out in a minimum of 100 mM NaCl.

3. TISSUE CULTURE

Suspension cultures of B95–8 cells, an Epstein Barr virus–positive lymphoid cell line, were maintained at concentrations of 4 x 10^5 to 2 x 10^6/ml in RPMI 1640 containing 10% heat–inactivated fetal calf serum and penicillin and streptomycin (all from Gibco) at 37°C in an humidified 5% CO_2 incubator. For the stability studies 200 µl of RPMI 1640 only, complete medium, or cell suspension was transferred to 96–well tissue culture dishes. Oligodeoxynucleotides were added to the wells to a final concentration of approximately 0.2 µM and were allowed to incubate at 37°C for times ranging up to 24 hours. Twenty–five µl samples were removed and incubated at 70°C for 5–10 min to curtail further degradation and were then stored at –20°C prior to gel migration analysis.

4. GEL MIGRATION ANALYSES

Marker phosphodiester oligodeoxynucleotides were 5'–labeled with ^{32}P using T4 polynucleotide kinase and purified using a spun column (Maniatis, et al., 1982) of Sephadex G–50 in H_2O. Annealing to nuclease–treated, complementary oligodeoxynucleotides was carried out at room temperature for 15 minutes prior to electrophoresis. Control annealing reactions were carried out in appropriate enzyme buffers. Samples were loaded onto a 20% acrylamide gel in 2.5% Ficoll 400. Gel electrophoresis was carried out at 4°C at 400 volts, 5–15 milliamps in TBE (89 mM Tris–Cl, 89 mM borate, 1 mM EDTA) buffer for 2 to 4 hours. For the tissue culture studies, appropriate amounts of samples with mixed with the ^{32}P–labeled oligodeoxynucleotide in

100 mM NaCl, 10 mM Tris–Cl (pH 7.6), and 1 mM EDTA. Annealing was carried out by heating to 65–70°C for 5 minutes followed by slow cooling to room temperature. These samples were loaded onto 20% polyacrylamide gels and run in TBE at a constant current (12 mA) with cold (5–10°C) circulating water. All gels were dried and examined by autoradiography.

5. IN VITRO TRANSCRIPTION

One µg of plasmid pSP65–ALA–D was digested with HindIII, NcoI, or PvuII and then extracted with phenol, extracted with chloroform:isoamyl alcohol (24:1), and ethanol precipitated. The pellet was washed with 70% ethanol, dried, and resuspended to 200 ng/µl in H_2O. The in vitro transcription reaction contained approximately 200 ng of linearized pSP65–ALA–D, 0.4 mM of all four cold ribonucleotide triphosphates, 20 µCi α–^{32}P–CTP (specific activity 800 Ci/mmol), 1 mM DTT, 40 mM Tris–HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine–[HCl_3] and 15 units of SP6 RNA polymerase, and was incubated for 1 hour at 37°C. Samples were extracted, precipitated and dried, and then resuspended in 20 µl H_2O.

6. RNASE H ANALYSES

One µl of resuspended transcript (approximately 40 ng of RNA) was annealed with from 5 to 100 ng (a large molar excess) antisense oligodeoxynucleotide, in 20 mM Tris–Cl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 100 µM DTT, and 5% (w/v) sucrose, in the presence of 10 units of RNasin, in a total volume of 9 µl. Following one minute incubation at 60°C, annealing

mixtures were incubated at room temperature (22°C) for 30 minutes. One µl (2 units) of E. coli RNase H (3.1.26.4) (BRL) was added and the reactions were incubated for 60 minutes at 37°C. One µl of 0.1 M EDTA was added to stop the reaction. Samples were extracted, ethanol precipitated, dried, and resuspended in 10 µl RNA loading buffer (50% formamide, 6.5% formaldehyde). Following incubation at 60°C for 5 minutes, samples were run on 6% denaturing (8 M urea) acrylamide gels at 350 volts, 45 mA, for 1 hour at room temperature. Results were visualized by exposing dried gels to film.

C. RESULTS

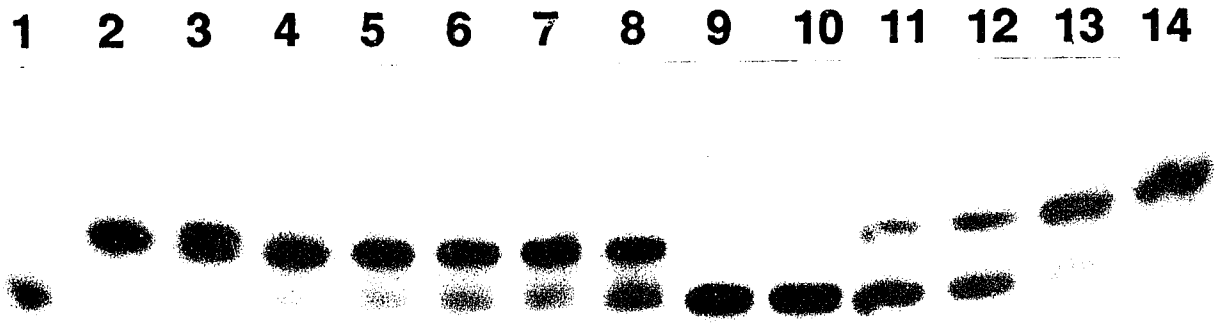
Analysis of nuclease sensitivity is based on the ability of an oligodeoxynucleotide to form a duplex with and gel shift a ^{32}P–labeled complementary oligodeoxynucleotide. The oligodeoxynucleotides used in these studies are presented in Table 5, where they are grouped into complementary sets designated as sense or antisense. Nuclease action by a given quantity of enzyme or degradation in tissue culture of substituted oligodeoxynucleotides was compared to results with equivalent quantities of control phosphodiester analogues. Figure 5 shows the gel migration analysis of a nuclease–treated oligodeoxynucleotide. Labeled 18–P–4, which served as the marker oligodeoxynucleotide (lane 1), had a lower mobility when hybridized to 14–P–3 (lane 2). Failure to decrease the mobility of (form a duplex with) the labeled complement indicates a sensitivity to the tested nuclease or the tissue culture condition.

TABLE 5: Oligodeoxynucleotides

SENSE		ANTISENSE	
SEQUENCE	**NAME**	**SEQUENCE**	**NAME**
GGATGCAGCTAAGTCAAG	18–P–4	CTTGACTTAGCTGCATCC	18–P–3
ATGCAGCTAAGTCA	14–P–4	TGACTTAGCTGCAT	14–P–3
AT<u>G</u>CA<u>G</u>CTAA<u>G</u>TCA	14–G–4	TG<u>A</u>CTT<u>A</u>GCTGC<u>A</u>T	14–A–3
A<u>T</u>GCAGC<u>T</u>AAG<u>T</u>CA	14–T–4	TGA<u>C</u>TTAG<u>C</u>TG<u>C</u>AT	14–C–3
		T<u>G</u>A<u>C</u>T<u>T</u>A<u>G</u>C<u>T</u>G<u>C</u>AT	14–Me^{6}a–3
		T<u>G</u>A<u>C</u>T<u>T</u>AG<u>C</u>TG<u>C</u>AT	14–Me^{6}b–3
		T<u>G</u>A<u>C</u>T<u>T</u>AGC<u>T</u>G<u>C</u>AT	14–Me^{5}a–3
		T<u>G</u>A<u>C</u>T<u>T</u>AGCTG<u>C</u>AT	14–Me^{5}b–3
CGCCATGCAGCCCCAGTC	18–P–2	TGGGGCTGCATG	12–P–1
		T<u>G</u>GGG<u>C</u>T<u>G</u>CATG	12–Me^{4}–1
		CT<u>GG</u>GG<u>C</u>T<u>G</u>CAT	12–Me^{5}a–1
		T<u>G</u>GG<u>G</u>C<u>T</u>G<u>C</u>A<u>T</u>G	12–Me^{5}b–1
		T<u>G</u>G<u>G</u>GC<u>T</u>G<u>C</u>ATG	12–Me^{6}–1
		T<u>GGGGCTGCATG</u>	12–Me^{10}–1

<u>N</u> = BASE WITH METHYLPHOSPHONATE 3'

FIGURE 5: Titration of Exonuclease Digestion



Lanes:

1	5'–^{32}P–labeled complementary oligodeoxynucleotide 18–P–4 (alone).		
2	Undigested 14–P–3 + 18–P–4 (control duplex)		
3 - 8	Spleen	(5' >——> 3')	5, 10, 15, 20, 25, 30 mU
9 - 14	Snake Venom	(3' >——> 5')	31.2, 15.6, 7.8, 3.9, 1.9, 0.9 μU

1. EFFECT OF EXONUCLEASES

Figure 5 depicts the digestion of control 14–P–3 by titrated quantities of spleen (lanes 3 – 8) and snake venom (lanes 9 – 14) exonuclease. The results of the studies of the action of these enzymes on the panel of oligodeoxynucleotides are summarized in Table 6A. All of the oligodeoxynucleotides tested with spleen exonuclease were >>200 times more resistant than their respective controls (half–time of 30 min), one of which (14–P–4) yielded an intermediate form during digestion. After 22 hours 14–C–3, 14–G–4, and 14–T–4 were apparently shortened but not completely digested, as evidenced by a slightly smaller gel shift (data not shown). The half–time for snake venom phosphodiesterase digestion of the control oligodeoxynucleotides was about 12 minutes for the enzyme concentration used. The oligodeoxynucleotide with the shortest relative half–life (14–G–4) had its first methylphosphonate bond as the third linkage in from the 3' end, while the other oligodeoxynucleotides tested had a methiphosphonate as the first or second linkage from the 3' end.

2. EFFECT OF ENDONUCLEASES

Figures 6A and B present visual comparisons of sensitivity of oligodeoxynucleotides to long DNase I treatment. Experiments with shorter timepoints allowed for the determination of digestion half–times for those oligodeoxynucleotides which were completely degraded by the long treatment. Table 6B presents the relative half–times for digestion of oligodeoxynucleotides by the endonucleases DNase I and DNase II. The half–time for digestion of

TABLE 6: Summary of Nuclease Sensitivities and Tissue Culture Stabilities

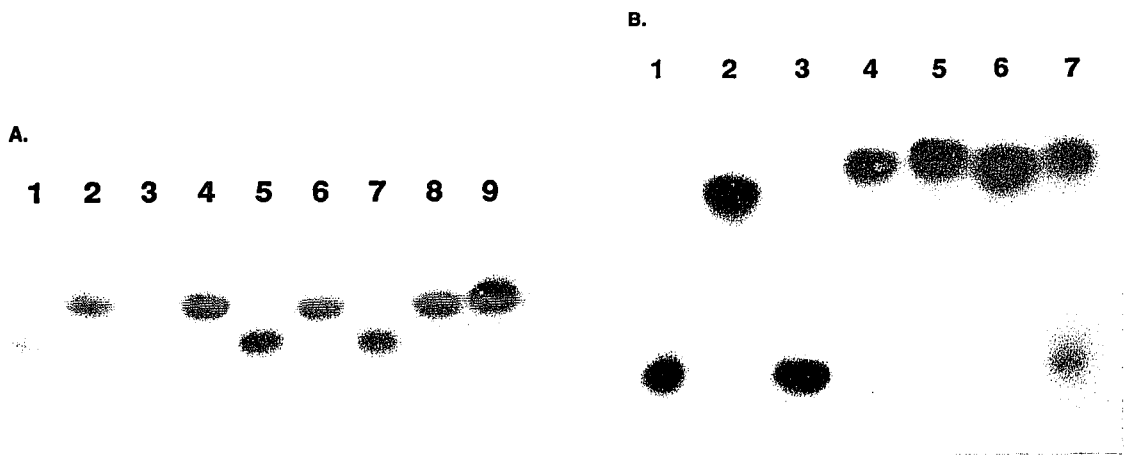
A.	RELATIVE HALF-LIFE	
EXONUCLEASES:	Spleen(5'->3')	Venom(3'->5')
14-P-3	1	1
14-P-4	3*	1
14-C-3	>>200	>500
14-G-4	>>200	100
14-A-3	>>200	>>500
14-T-4	>>200	>500
14-Me^{6}a-3	>>200	>>500

B.	RELATIVE HALF-LIFE	
ENDONUCLEASES:	DNaseI	DNaseII
14-P-3	1	1
14-A-3	1	>5
14-C-3	6	2
14-Me^{5}b-3	12	>5
14-Me^{5}a-3	300	>5
14-Me^{6}b-3	>600	>5
14-Me^{6}a-3	>600	>5

C.	RELATIVE HALF-LIFE		
CULTURE CONDITION:	RPMI	+FCS	+FCS, B95-8
14-P-3	STABLE	1	1
14-Me^{5}b-3	"	30	30
14-Me^{5}a-3	"	30	30
14-Me^{6}b-3	"	30	30
14-Me^{6}a-3	"	30	30

* Intermediate form was detected

FIGURE 6: DNase I Sensitivity



A. All lanes: 5'-^{32}P-labeled complementary oligodeoxynucleotide 18-P-4.
Lanes 2,4,6,8: Untreated Lanes 3,5,7,9: DNase I treated

Lanes: Oligodeoxynucleotide tested

1 None (18–P–4 alone).

2 - 3 14–P–3

4 - 5 14–A–3

6 - 7 14–C–3

8 - 9 14–Me^{6}a–3

B. All lanes: 5'-^{32}P-labeled complementary oligodeoxynucleotide 14-P-4.
Lanes 2,4,6: Untreated Lanes 3,5,7: DNase I treated

Lanes: Oligodeoxynucleotide tested

1 None (14–P–4 alone).

2 - 3 14–P–3

4 - 5 14–Me^{6}b–3

6 - 7 14–Me^{5}a–3

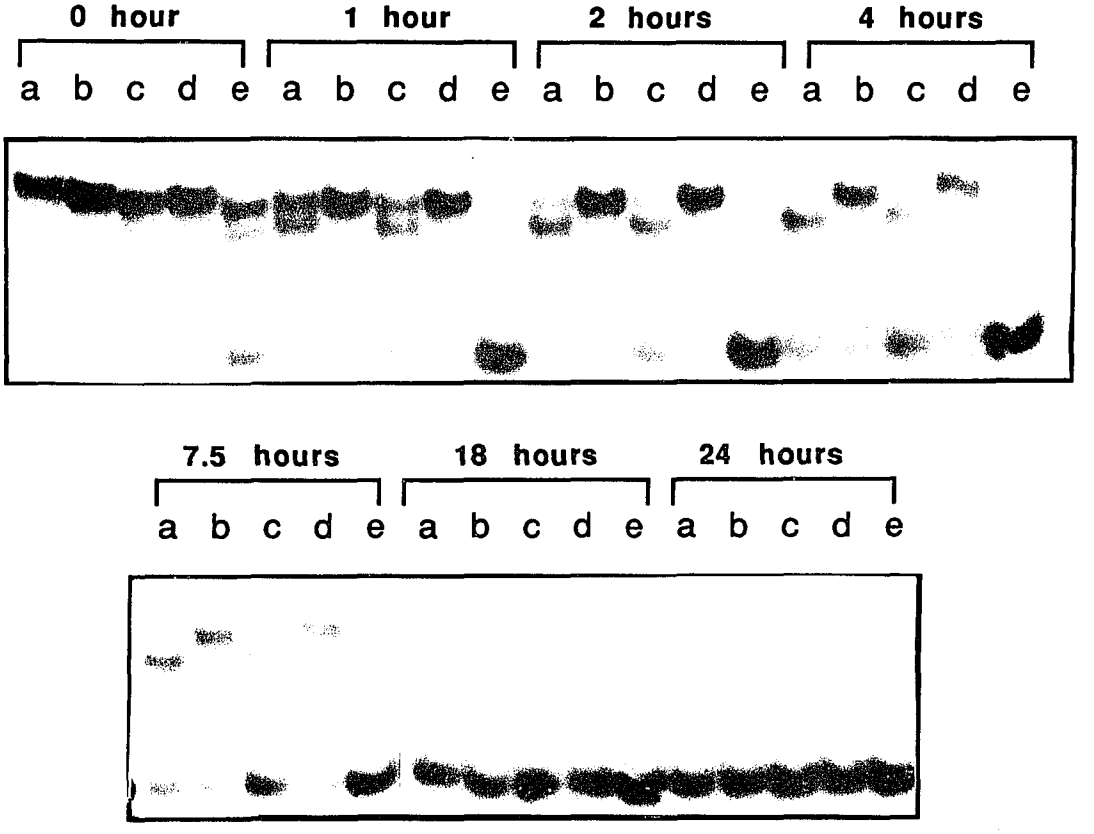
control 14–P–3 by DNase I was on the order of 10 minutes. 14–A–3, which has an internal span of five phosphodiester linkages, is as sensitive to DNase I as the control 14–P–3. 14–Me^{6}a–3 has alternating diester and methylphosphonate linkages and was over 600 times more resistant to DNase I than 14–P–3. The general trend is that as the phosphodiester span is decreased, the oligodeoxynucleotide becomes more resistant to endonuclease activity. Given a time frame for the nuclease studies of 24 hours and the fact that the DNase II reactions were much slower than those for DNase I (half–time for 14–P–3 digestion of 10 hours), the most resistant methylphosphonates oligodeoxynucleotides can only be designated as >5 times more stable than the control.

3. STABILITY OF OLIGODEOXYNUCLEOTIDES IN TISSUE CULTURE

Table 6C summarizes the results of the tissue culture studies. All of the oligodeoxynucleotides tested were completely stable to 37°C incubation for 24 hours in RPMI 1640 alone. The results of the gel analysis showed that the 0 hour samples and the 24 hour samples were identical to each other and to the 0 hour samples shown in Figure 7, except that 14–P–3 (sample e) showed no evidence of degradation (no hybrid doublet) in RPMI alone. In contrast, all of the oligodeoxynucleotides were degraded by incubation in either complete medium or in cell suspensions. The similar degradation in either complete medium or cell suspensions indicates that the enzymatic activity leading to degradation is associated with the serum.

Figure 7 presents the results of stability testing in cell suspensions. The phosphodiester oligodeoxynucleotide 14–P–3 had a half–life of less than 60

FIGURE 7: Stability in Cell Suspension



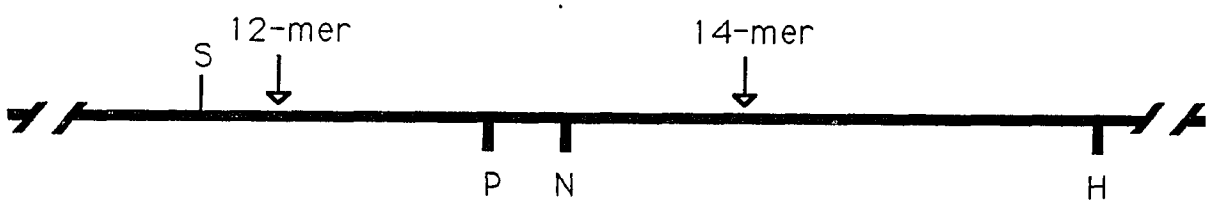
Oligodeoxynucleotides were incubated in suspensions of B95–8 cells in complete RPMI 1640 medium. Samples were taken at the indicated times and were treated as described in Materials and Methods. The tested oligodeoxynucleotides were a) 14–Me^{6}a–3, b) 14–Me^{6}b–3, c) 14–Me^{5}a–3, d)14–Me^{5}b–3, and e) 14–P–3. The ^{32}P–labeled complementary oligodeoxynucleotide was 14–P–4.

minutes in cell suspensions (or in complete medium). Results from similar experiments with shorter timepoints suggest a half–life of approximately 15 minutes. The oligodeoxynucleotides with methlyphosphonate substitutions were significantly more stable than their phosphodiester analogue, but they all displayed relatively similar half–lives of approximately 7.5 hours, regardless of the arrangement of the methylphosphonate linkages. It appears from the autoradiogram that 14–Me^{6}a–3 (alternating modifications) was the most stable, and the oligodeoxynucleotides containing 2, 3 and 4 contiguous phosphodiester bonds were nearly identical in stability. A 3'–exonucleolytic activity was evidenced by single step–wise cleavage of oligodeoxynucleotides 14–Me^{6}a–3 and 14–Me^{5}a–3, both of which contain 3'–phosphodiester–linked thymidine residues, and the absence of cleavage of oligodeoxynucleotides 14–Me^{6}b–3 and 14–Me^{5}b–3, which contain 3'–methylphosphonate–linked thymidine residues. However, a fully methylphosphonate–substituted oligodeoxynucleotide (12–Me^{10}–1) was stable for 24 hours with no apparent diminution of hybridizable material (data not shown).

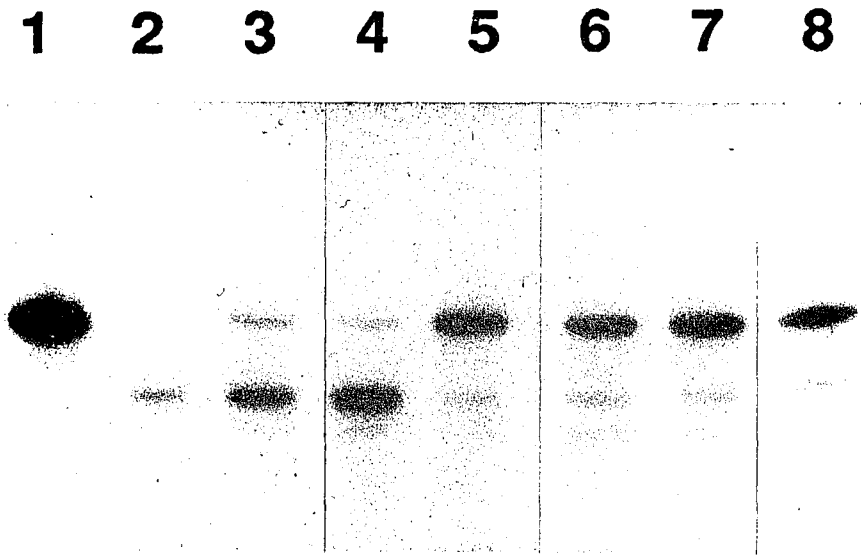
4. PARTIALLY METHYLPHOSPHONATE–SUBSTITUTED OLIGODEOXYNUCLEOTIDES CAN SERVE AS RNASE H SUBSTRATES

^{32}P–labeled runoff transcripts were made from pSP6–ALA–D (Figure 8A) and incubated with various oligodeoxynucleotides in the presence of RNase H. Using a HindIII runoff transcript, the methylphosphonate–substituted oligodeoxynucleotides 14–A–3 and 14–C–3 served as RNase H substrates, while 14–Me^{6}a–3 did not, even up to a concentration of 10 μg/ml (data not shown). The 14–mer binding site appeared to be a poor site for RNase H

FIGURE 8: RNase H Assay



A: Map of pSP65-ALA-D Arrows indicate oligodeoxynucleotide binding sites. Restriction endonuclease sites for runoff transcription: P = PstI, N = NcoI, H = HindIII. S = start of transcription.



B: RNase H Assay

Lanes: Tested oligodeoxynucleotides:

1 RNA transcript (501 nt)

2 12–P–1, control phosphodiester [386 nt product (>——>)]

3 - 4 12–Me^4–1

5 - 6 12–Me^5b–1

7 - 8 12–Me^6–1

Lanes 4,6,8 contained 4–fold more oligodeoxynucleotide than lanes 3,5,7.

digestion since the use of the control phosphodiester oligodeoxynucleotide 14–P–3 only allowed for cleavage of a small amount of the original transcript material, perhaps due to secondary structure in the RNA at this region. The results of the RNase H studies using methylphosphonate–substituted oligodeoxynucleotides and the phosphodiester control complementary to the 12–mer binding site (Figure 8A) are shown in Figure 8B. The NcoI runoff transcript (501 nt) (lane 1), is completely cleaved by RNase H in the presence of 12–P–1 (lane 2). 12–Me^4–1 is composed of alternating methylphosphonate and diester linkages with an internal span of 3 phosphodiesters, and it permits cleavage of approximately 75% of the RNA (lanes 3 and 4). Comparable results were obtained with oligodeoxynucleotide 12–Me^5a–1, which has an internal span of 2 diesters and 2 diesters at the 5' end (data not shown).12–Me^5b–1, with a span of 2 diesters, and 12–Me^6–1, which has alternating linkages throughout, are poor RNase H substrates (lanes 5 through 8). The four–fold increase in oligodeoxynucleotide concentration does not increase the RNA cleavage, indicating that addition of more oligodeoxynucleotide does not increase the amount of available RNase H substrate.

D. DISCUSSION

The antisense effects previously reported with fully methylphosphonate–substituted oligodeoxynucleotides required relatively high concentrations (Smith, et al., 1986; Agris, et al., 1986; Sarin, et al., 1988). A major reason for this requirement may be the isomeric nature and destabilizing effect of each modified linkage, leading to a decrease in duplex stability (Bower, et al., 1987; Quartin & Wetmur, 1989). In addition, since RNase H will not cleave the RNA

in a duplex with fully–substituted molecules (Maher & Dolnick, 1988), the effect seen was probably due to a physical block of the translational machinery, and this mechanism alone may not be very effective. Partial substitution by methylphosphonates would maximize duplex stability. It is of interest to create a balanced combination of phosphodiester and methylphosphonate linkages that will confer nuclease resistance while permitting cleavage of the RNA in the oligodeoxynucleotide:RNA hybrid.

We have examined the questions of sensitivity to specific nucleases and stability in a cellular environment by comparing partially methylphosphonate–substituted oligodeoxynucleotides to phosphodiester analogues. Our method of gel shift analysis to assess oligodeoxynucleotide stability is particularly appropriate where such oligodeoxynucleotides are being considered for use as antisense agents, since the assay directly tests the ability of an oligodeoxynucleotide to hybridize with its complement. The results of our exonuclease experiments indicate that the presence of methylphosphonate linkages causes a considerable decrease rather than a complete block to the action of exonucleases. This suggests that exonucleolytic attack on methylphosphonate–substituted oligodeoxynucleotides may be similar to that found for snake venom phosphodiesterase digestion of DNA containing thymidine dimers, where the enzyme slowly breaks the internal bond of the base beyond each encountered dimer (Laskowski, 1971). It has been reported that two consecutive methylphosphonate linkages served to increase half–times of digestion by both snake venom and spleen phosphodiesterase by two orders of magnitude, as determined by HPLC analysis of digest products (Agrawal & Goodchild, 1987). We find from our gel migration analyses that the presence of just one methylphosphonate linkage near the 5' and 3' ends of

an oligodeoxynucleotide causes an increase in digestion half–times by more than two orders of magnitude.

While our studies with DNase I indicate resistance to endonuclease increases with decreasing internal spans of phosphodiester linkages, methylphosphonate oligodeoxynucleotides with internal contiguous phosphodiester linkages ranging from 1 to 4 have relatively uniform half–lives of 7.5 hours when incubated in complete medium or with cultured cells. The initial 3'–exonucleolytic cleavage seen with two of the oligodeoxynucleotides and the failure to observe subsequent step–wise degradation of any of the oligodeoxynucleotides is not understood at this time. A single endonucleotlytic cleavage in the center of the oligomers could generate such a degradative pattern. Alternatively, modification of a nucleoside or depurination and subsequent cleavage of the sugar–phosphate or sugar methylphosphonate backbone could also generate these results. It is interesting to note that Wickstrom (1986) finds that phosphodiester oligodeoxynucleotides are completely stable to incubation for up to 2 hours in medium with 5% fetal calf serum (Sigma), but were degraded in bovine calf serum (Hyclone), whereas we find that the presence of fetal calf serum (Gibco) is the basis of oligodeoxynucleotide degradation.

We have demonstrated that partially methylphosphonate–substituted oligodeoxynucleotides with 3 or more contiguous phosphodiester linkages will hybridize with complementary RNA and promote cleavage of the RNA by RNase H. Thus it is possible to synthesize an antisense oligodeoxynucleotide with methylphosphonate and phosphodiester linkages that will have increased stability over a phosphodiester oligodeoxynucleotide in tissue culture, and perhaps in vivo, as well as have the ability to promote cleavage of target RNA.

IV.

BRANCH MIGRATION–MEDIATED DNA CLONING

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A. INTRODUCTION

The cloning or recloning of a particular DNA fragment from a eukaryotic organism is normally carried out by the two-step process of library construction and library screening (Maniatis, et al., 1982). The work required could be greatly reduced if a library could be constructed which was initially highly enriched for the particular fragment. The determination of the size of a particular DNA restriction fragment in a mixture of fragments is normally carried out by the two-step process of gel electrophoresis and Southern blot (Southern, 1975) hybridization. A simpler procedure would be to label the particular fragment prior to electrophoresis. In this communication, we describe a branch-migration study aimed at achieving these ends.

More than 20 years ago, experiments with homopolymers demonstrated that polybromodeoxycytidine would displace polydeoxycytidine from a duplex with polydeoxyinosine (Michelson, et al., 1967). Other similar displacement reactions have been carried out where the displacing strand formed a more stable duplex than the displaced strand. RNA will displace DNA in concentrated formamide leading to the formation of an R-loop (Thomas, et al., 1976). The reverse reaction, which is favored in aqueous solution, has been exploited for the development of a homogeneous nucleic acid hybridization assay (Vary, 1987).

Displacement of one DNA strand by another requires energy. DNA strands will displace identical DNA strands from superhelical molecules leading to the formation of D-loops. The reverse reaction, driven by loop free energy, may be initiated by removing the topological constraint (Radding, et al., 1977). In an analogous reaction driven by loop free energy, a short strand forming a

duplex within a long single strand was easily displaced by a longer, overlapping strand during DNA reassociation (Green & Tibbetts, 1981). Recently, RecA protein, using ATP as an energy source, was employed to reverse DNA branch migration reactions, permitting the formation of D–loops in linear molecules (Rigas, et al., 1986).

The branch migration procedure we have developed uses displacer bromodeoxycytidine–containing oligodeoxynucleotides. This method leads to the specific attachment of both the displacer oligodeoxynucleotide and a partially complementary linker oligodeoxynucleotide to the end of a particular DNA fragment containing a sequence complementary to the displacer oligodeoxynucleotide. This specific attachment may be used (A) to label a particular fragment for detection without blotting and subsequent hybridization, (B) to mark a particular fragment for affinity chromatography, or (C) to facilitate cloning by introducing a new 5' or 3' overhang compatible with a restriction endonuclease site in a cloning vector.

B. MATERIALS AND METHODS

1. OLIGODEOXYNUCLEOTIDES, PLASMIDS AND ENZYMES

All oligodeoxynucleotides (Table 7) were synthesized on an Applied Biosystems Model 380B DNA Synthesizer using standard phosphoramidite chemistry. Bromodeoxycytidine incorporation was accomplished using a 5–bromodeoxycytidine phosphoramidite monomer (ABN–Fisher). Purification steps were limited to hydrolysis of base–protecting groups and cleavage from the support with NH_4OH, evaporation, resuspension and ethanol precipitation.

TABLE 7: Oligodeoxynucleotides

T_m Analysis

14-dC-S	ATG CAG CTA AGT CA
14-dC-A	TGA CTT AGC TGC AT
14-BrdC-S	ATG CAG CTA AGT CA
14-BrdC-A	TGA CTT AGC TGC AT

T_m and Blunt-end Displacement

12-dC-S	CAT GCA GCC CCA
12-dC-A	TGG GGC TGC ATG
12-BrdC-S	CAT GCA GCC CCA

Dangling-end Displacement

12-dC-A	TGG GGC TGC ATG
16-dC-A	TGG GGC TGC ATG GCG T
16-BrdC-S	ACG CCA TGC AGC CCC A

Capture

14-dC-L	CAT CAT CAT CCA TG
34-dC-D	GAT GAT GAT GTG CAG CCA ATG CCC CAG GAG CCC T
34-BrdC-D	GAT GAT GAT GTG CAG CCA ATG CCC CAG GAG CCC T
34-BrdC-D-E@10	GAT GAT GAT GTG CAG CCA AAG CCC CAG GAG CCC T
34-BrdC-D-E@24	GAT GAT GAT GTG CAG CCA ATG CCC CAG GAG CCC A

C = 5'-Bromodeoxycytidine

All oligodeoxynucleotides were 5'–labeled with ^{32}P using T4 polynucleotide kinase, subjected to polyacrylamide gel electrophoresis on 20% acrylamide–8M urea gels, and visualized by autoradiography. A single oligodeoxynucleotide species of the correct size was routinely detected. Plasmid pALA–D is a pUC9 expression vector containing the cDNA sequence of human δ–aminolevulinate dehydratase (ALA–D), a heme biosynthetic enzyme (Wetmur, et al., 1986). pALA–D and pUC19 were each propagated in E. coli strain HB101 and purified by standard methods (Maniatis, et al., 1982). Restriction endonucleases, T4 polynucleotide kinase and T4 DNA ligase were obtained from and used as recommended by New England Biolabs.

2. MELTING TEMPERATURE ANALYSES

Melting temperatures for oligodeoxynucleotides were determined as previously described (Quartin & Wetmur, 1989).

3. DISPLACEMENT ASSAY

A gel migration assay was used to monitor the displacement of 5'–^{32}P–labeled dC–containing oligodeoxynucleotides from unlabeled complementary oligodeoxynucleotides by their BrdC–containing analogues. Labeled dC–containing oligodeoxynucleotides were annealed to unlabeled dC–containing complementary strands at room temperature in 1M NaCl at concentrations of 1 and 3 μg/ml, respectively, and then brought to 4°C. BrdC–containing analogues of the labeled strands (at concentrations ranging from 3 to 400 μg/ml) were incubated with dC–containing duplexes as a function of time at

various temperatures. Aliquots taken at each time point were stored at –20°C prior to electrophoresis. Samples were loaded onto a 20% acrylamide gel in 2.5% ficoll 400, and electrophoresis was performed at 4°C at 400 volts, 5–15 milliamps in 89mM TrisCl, 89mM borate, 1mM EDTA, pH 8 (TBE). The gels were dried, and the results were obtained by autoradiography.

4. CAPTURE AND CLONING PROCEDURES

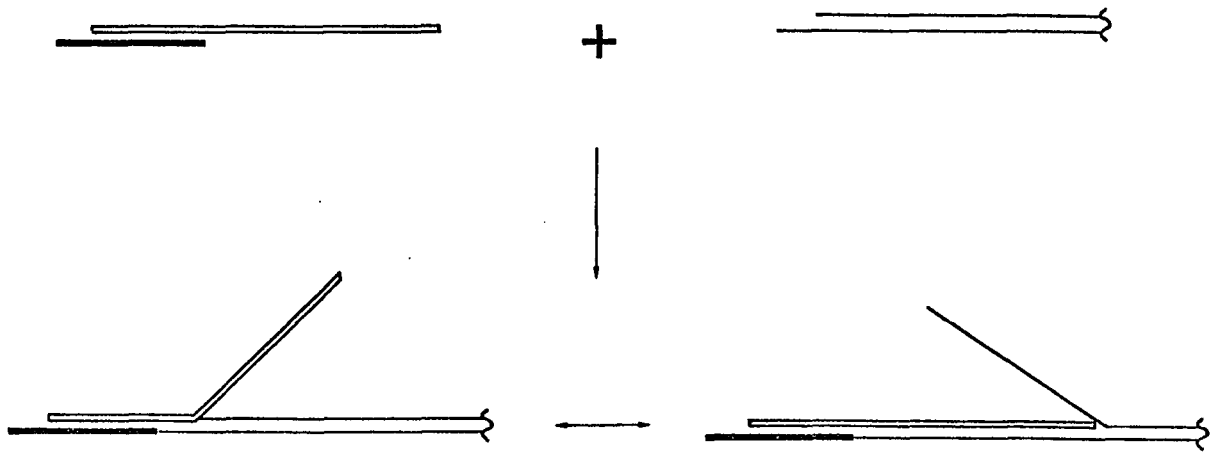
A 14–mer linker oligodeoxynucleotide was ^{32}P–labeled using T4 polynucleotide kinase. The reaction with labeled ATP was followed by reaction with excess unlabeled ATP to assure that all linker strands contained a 5'–phosphate. The linker oligodeoxynucleotides were purified over spun columns (1) of Sephadex G–50. Primary ligation (capture): Restriction endonuclease–digested pALA–D (PstI/RsaI or PstI/SmaI) (20μg/ml) was mixed with BrdC–containing 34–mer displacer oligodeoxynucleotide (6μg/ml) and 5'–^{32}P–labeled 14–mer linker oligodeoxynucleotide (2μg/ml) in a pH–reduced T4 DNA ligase buffer (50mM Tris–HCl pH 7.0, 1mM ATP, 10mM $MgCl_2$, 20mM DTT, 50μg/ml bovine serum albumin), incubated at 55°C for 10 minutes, cooled to room temperature, and incubated at 16°C for from 1 minute to 20 hours in the presence of from 5 to 500U/ml of T4 DNA ligase. All units of ligase are New England Biolabs units. Ligation products were examined on ethidium bromide–containing, 1% agarose gels by UV illumination and by autoradiography. Secondary ligation: Restriction endonuclease–digested pUC19 (PstI/SphI) was added to 10μl of the primary ligation mixture to a final concentration of 10μg/ml in the same ligation buffer and incubated with 20,000U/ml T4 DNA ligase at 16°C for 20 hours. E. coli strain DH5α was used

for the cloning experiments. Transformation (rubidium chloride) and identification of ß–galactosidase–negative clones were carried out according to previously described techniques (Maniatis, et al., 1982). Double–stranded DNA sequencing was carried out with Sequenase (United States Biochemicals, Inc.), according to the protocol of the manufacturer, using both miniprep and large–scale alkaline lysis/CsCl–banded plasmid DNA.

C. THEORETICAL

The basic experiment is outlined in Fig. 9. Assume that the branch migration event is initiated by forming four base pairs with a 3'–overhang remaining after cleavage of DNA with a restriction endonuclease such as PstI. These base pairs are numbered 1 through 4. Additional base pairs would form only at the expense of existing base pairs, a branch migration event. For the purposes of this calculation, assume that the free energy of any structure involving two single–stranded branches is independent of the location of the branch point. Several calculations are presented below. Case I occurs when the PstI site overhang (positions 1 through 4) and a limited number of additional bases (positions 5 to m) in the displacer strand are identical, or equivalent, to the bases to be displaced. Case II occurs when the sequence of the remainder of the displacer strand is identical, or equivalent, to the displaced strand, with potential base pairs 1 through n. Case III occurs when a non–identical (or non–equivalent) base is introduced into the displacer strand.

FIGURE 9



Branch migration of displacer (D), bound to linker (L), oligodeoxynucleotide into a recipient (R) duplex. Upper left: duplex of D (open rectangle) and L (filled rectangle). Upper right: R duplex with 4 base 3'–overhang. Lower: conversion between the D–L duplex bound form (left) and the branch migrated form (right).

Let q = partition function.

σ = initiation parameter (includes base stacking at a single strand break).

C = oligodeoxynucleotide concentration (molar).

s_k = equilibrium constant to add base k to a stack; s >> 1 (ignore fraying).

Ω = relative stability of 2 versus 1 single-stranded branch.

K = relative stability of BrdC–dG base pair versus dC–dG base pair.

B_k = 1 if branch migration results in no nucleotide substitution.

B_k = K if branch migration replaces dC by BrdC.

μ = relative stability of a mismatched base pair.

Case I: Incomplete branch migration: A PstI site (positions 1 through 5) and additional (6 through m) branch migration but without completion:

$$q_1 = 1 + \sigma C\{\prod_{k=1}^{4} s_k [1 + \Omega \sum_{i=5}^{m} \prod_{k=5}^{i} B_k]\}$$

Case II: Complete branch migration to position n:

$$q_2 = 1 + \sigma C \{\prod_{k=1}^{4} s_k [1 + \prod_{k=5}^{n} B_k + \Omega \sum_{i=5}^{n-1} \prod_{k=5}^{i} B_k]\}$$

Case III: Branch migration with a single mismatch at site j costing μ:

$$q_3 = 1 + \sigma C \{\prod_{k=1}^{4} s_k [1 + \mu \prod_{k=5}^{n} B_k + \Omega ((\sum_{i=5}^{j-1} \prod_{k=5}^{i} B_k) + (\mu \sum_{i=j}^{n-1} \prod_{k=5}^{i} B_k))]\}$$

Assuming that the majority of all unligated sites lack bound D–L duplexes, the ratio of uptake (ligation) of oligodeoxynucleotides at any two sites is given by:

$$r_i / r_j = (q_i - 1) / (q_j - 1)$$

Take a PstI site followed by no additional similarity to be the standard: $r_j = r_1$ (m = 5).

$$r_1 \text{ (all m)} \; / \; r_1 \text{ (m = 5)} \; = \; (1 \; + \; \Omega \sum_{i=5}^{m} \prod_{k=5}^{i} B_k) \; / \; (1 + \Omega)$$

$$r_2 \; / \; r_1 \text{ (m = 5)} \; = \; (1 \; + \; \prod_{k=5}^{n} B_k \; + \; \Omega \sum_{i=5}^{n-1} \prod_{k=5}^{i} B_k) \; / \; (1 + \Omega)$$

$$r_3 \; / \; r_1 \text{ (m = 5)} = \; [1 \; + \; \mu \prod_{k=1}^{n} B_k \; + \; \Omega((\sum_{i=5}^{j-1} \prod_{k=5}^{i} B_k) \; + (\mu \sum_{i=j}^{n-1} \prod_{k=5}^{i} B_k))] \; / \; (1 + \Omega)$$

D. RESULTS

1. DISPLACEMENT OF dC–CONTAINING HOMOLOGUES BY BrdC–CONTAINING OLIGODEOXYNUCLEOTIDES

The oligodeoxynucleotides used for melting temperature (t_m) analyses are listed in the first two sections of Table 7. Table 8a presents the t_m values for dC–containing oligodeoxynucleotides and for their BrdC–containing analogues. The substitution of BrdC for dC nucleotides causes an increase in duplex stability, which results in a higher t_m. The increase in t_m, ΔT_m, can be related to the change in free energy due to N_{BrdC} nucleotides ($\Delta G°_d$) by:

$$\Delta G°_d \; = \; \frac{T°\Delta H°}{T_m T_m'} \Delta T_m \; / \; N_{BrdC}$$

where T° is 298.16°K, the helix–coil transition enthalpy, $\Delta H°$, is 98,800 Kcal/mol (Quartin and Wetmur, 1989), T_m is the dC oligodeoxynucleotide melting temperature, and T_m' is the melting temperature for the BrdC analogue. The average value for $\Delta G°_d$ derived from the data in Table 8a is 0.4 kcal/mole at pH 7. Thus, $-RT \ln K = 400$ or $K = 1.9$ (see Theoretical definitions). The

TABLE 8: Bromodeoxycytidine Thermodynamics and Displacement Reactions

a. THERMODYNAMICS:		**t_m in 1 M Na^+, C = 6 μM**		
Oligodeoxynucleotides		**pH 7**	**pH 4**	**pH 10**
14–dC–A + 14–dC–S		57°		
14–dC–A + 14–BrdC–S		63°		
14–dC–S + 14–BrdC–A		62.5°		
14–BrdC–A + 14–BrdC–S		65°		
12–dC–A + 12–dC–S		60°	53.5°	50°
12–dC–A + 12–BrdC–S		69.5°	70°	54°
Conclusion:	$\Delta G°_d$ =	0.4	0.68	0.17 kcal/mole.

b. KINETICS: Half time for displacement (minutes)

Blunt 12–dC–S* (C=0.25μM) + 12–dC–A (C=0.75μM)

	Displacer 12–BrdC–S at		
	101μM	**20μM**	**4μM**
°C			
37	2	4–8	16–32
32	4–8	8–16	32
27	4–8	32–64	128–256

Overhang 12–dC–S* (C=0.25μM) + 16–dC–A (C=0.57μM)

	Displacer 16–BrdC–S at	
	3μM	**0.57μM**
°C		
47	<1	4–8
37	<1	16–32
27	<1	2–4
22	<1	<1

* 5'–^{32}P–labeled

FIGURE 10

Displacement of a dC oligodeoxynucleotide by its BrdC analogue in 1 M NaCl. Lane 1: 5'–^{32}P–labeled 12–dC–S only. Lane 2: with 12–dC–A. Lanes 3–4: 12–dC–S/12–dC–A duplex incubated at 32°C with 400µg/ml 12–BrdC–S for 128 min (lane 3) and 256 min (lane 4). Lanes 5–13: the same duplex incubated with 80µg/ml 12–BrdC–S for 1, 2, 4, 8, 16, 32, 64, 128, and 256 min, respectively.

value of $\Delta G°_d$ (and hence K) is pH–dependent because of the difference between the acid–base dissociation constants of BrdC and dC.

BrdC–containing oligodeoxynucleotides were examined for their ability to displace their dC–containing analogues from duplexes. The oligodeoxynucleotides used in these studies appear in the second and third sections of Table 7. Fig. 10 lanes 5 through 13 shows the time dependence of the displacement of 5'–^{32}P–labeled 12–dC–S from the blunt–ended 12–dC–S:12–dC–A duplex by 80μg/ml 12–BrdC–S. Lanes 1 and 2 show 12–dC–S alone (non–shifted), and in a duplex (shifted), respectively. Table 8b presents a summary of displacement rates. The reactions initiated at blunt ends proceed faster as the temperature increases due to increased breathing at the blunt ends. Displacement reactions where initiation occurred at 4 base overhangs were more than two orders of magnitude faster than reactions initiated at blunt ends. The stability of a duplex formed between the displacing strand and the overhang of a preformed duplex increases with decreasing temperature. Thus, in the temperature range of 22 to 37°C, the rate of displacement decreases with increasing temperature. Apparently, at 47°C the displacement reaction proceeds by a different mechanism, perhaps involving the dissociation of the preformed duplex due to the proximity of its melting temperature.

2. BRANCH MIGRATION–MEDIATED CAPTURE OF LINKER OLIGODEOXYNUCLEOTIDES

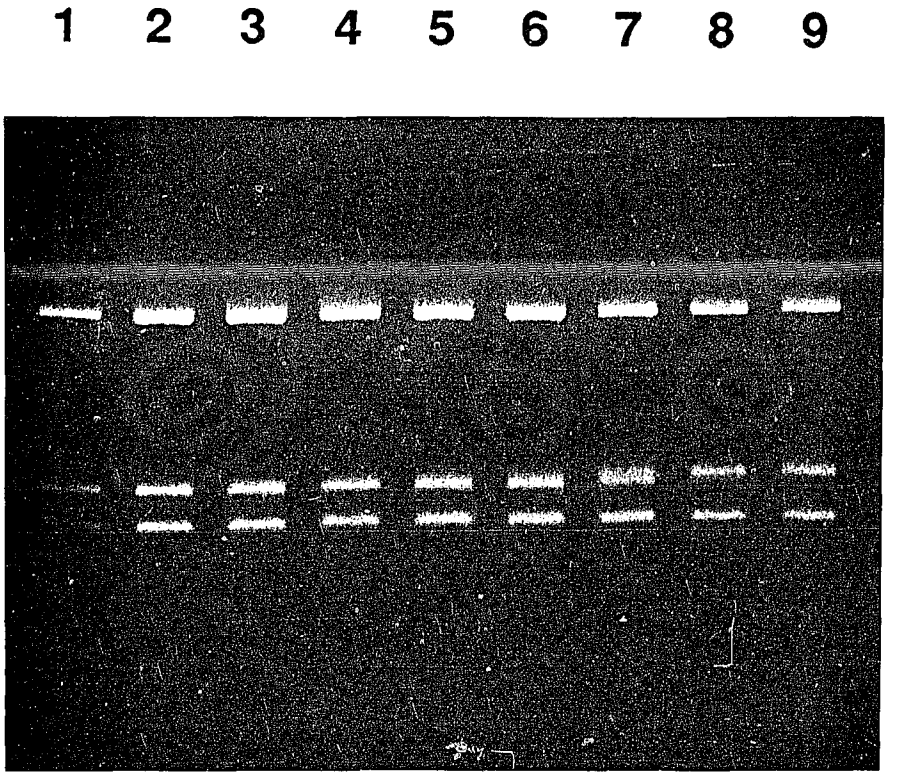
The basic experiment is depicted in Fig. 9. The same 5'–^{32}P–labeled linker, 14–dC–L, was used throughout. The standard displacer molecule, 34–

BrdC–D, contained BrdC in place of dC and was capable of forming a perfect duplex with the complementary strand in a recipient duplex. Plasmid pALA–D, when digested with RsaI/PstI, yields seven fragments four of which contain one PstI end with a 3'–overhang and one blunt RsaI end. Fig. 11, lane 1 depicts such a digest. The third largest visible band is a 694 nt fragment with two blunt ends. The second largest of these fragments, 720 nt, contains the complementary sequence to 34–BrdC–D. Thus, uptake of the L–D duplex by this R (recipient) duplex is described by Case II in Theoretical. The other three fragments having a 3'–overhang, 1807, 308, and 71 nt, the latter of which is not visible in Fig. 11, contain limited complementarity to 34–BrdC–D in addition to the four bases of the PstI overhang. The additional complementarity in the 308 nt R duplex is limited to the single remaining base in the PstI site. The 1807 nt R duplex contains four additional complementary base pairs. Thus, uptake of the L–D duplex by the 1807 and 308 nt R duplexes is described by Case I in Theoretical with m equal to 9 and 5, respectively.

Fig. 11, lanes 2 through 9, demonstrates ligation of the pALA–D fragments in the presence of the displacer 34–BrdC–D (6μg/ml) and the linker 14–dC–L (2μg/ml) with 5 U/ml ligase. Ligation results in a small decrease in the mobility of the 720 nt band. This reaction is 50% completed in 32 min. A similar experiment carried out using 25 U/ml ligase proceeded 50% to completion in 8 min (data not shown). Thus, the reaction rate was linearly dependent on ligase concentration.

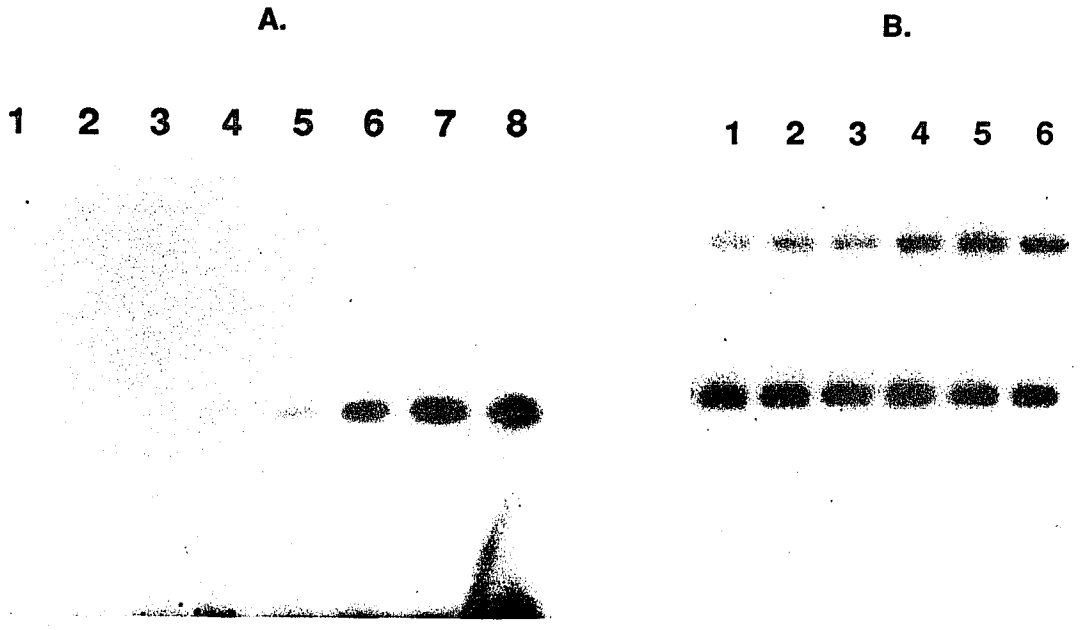
Fig. 12A is an autoradiogram of Fig. 11. Under conditions where the reaction with the 720 nt band is carried to completion, the 1807 nt band (m=9) is barely visible and the 308 nt band (m=5) cannot be detected. These results

FIGURE 11



Capture reaction of 34–BrdC–D plus 14–dC–L. UV fluorogram of 1% agarose gel. Lane 1: RsaI/PstI digested pALA–D (200ng); visible bands are 1807, 720, 694 and 308 nt. Lanes 2–9: products following ligation in the presence of 34–BrdC–D (6μg/ml), 14–dC–L (2μg/ml), and 5U/ml ligase for 1, 2, 4, 8, 16, 32, 64, and 128 min, respectively.

FIGURE 12



A: Autoradiogram of Fig. 11.

B: Same as Fig. 12A, except 500U/ml ligase.

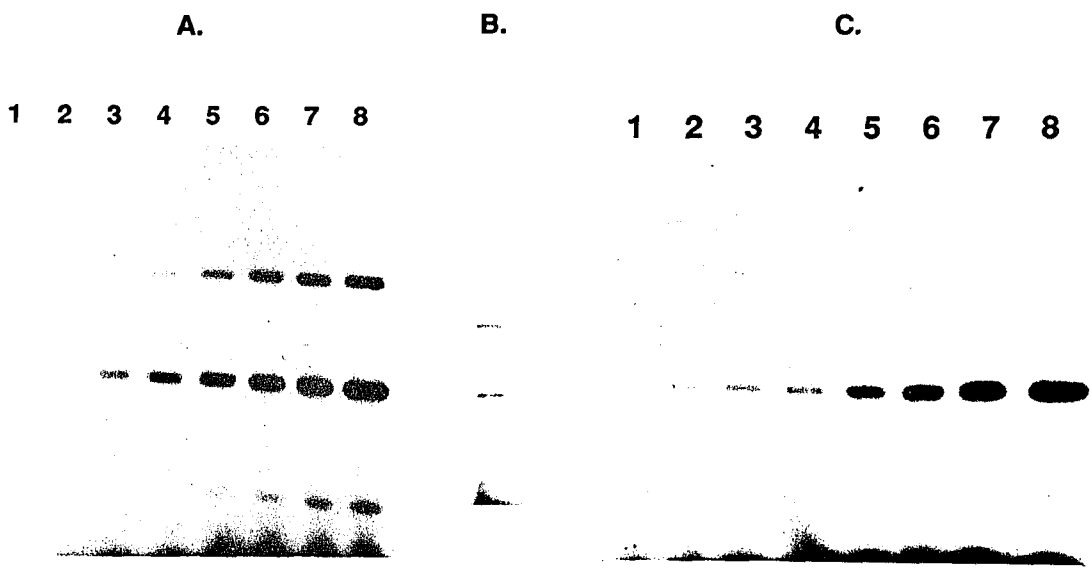
demonstrate conditions for maximum specificity of linker capture. Fig. 12B presents the results of ligation in the presence of 500 U/ml ligase. Under these conditions, the capture of the linker by the 1807 nt band, although proceeding more slowly, tended toward completion, and some linker capture by the 308 nt band was detected.

Fig. 13A shows an autoradiogram analogous to Fig. 12A, where the displacer strand is 34–dC–D instead of 34–BrdC–D. Under conditions where the reaction with the 720 nt band is carried 50% to completion, based on the fluorogram of the gel (data not shown), both the 1807 nt band (m=9) and the 308 nt band (m=5) were easily detected. Table 9a contains relative autoradiographic intensities of the 1807, 720 and 308 nt bands, using 34–BrdC–D and 34–dC–D as displacer molecules. The intensities of the 308 nt bands were assigned the value of 1. Since the values for the 1807 and 720 nt bands were determined using a 2–fold time course assay, they are reliable to ±50%. The data in Table 9a demonstrate that the use of BrdC is important for obtaining highly–specific Case II reactions.

Calculated intensity ratios in Table 9 were obtained using K = 1.7 at 16°C and $\Omega = 3$. The value of 1.7 was chosen as a best fit to the 34–BrdC–D data and was consistent with the ratio of 1.9 found in 1 M NaCl at 60°C. The results were insensitive to the choice of Ω as long as $\Omega \geq 2$. A value of $\Omega \geq 1$ implies that a structure with two single–stranded branches is more stable than a structure with one branch.

Fig. 13B and 13C depict Type III reactions where mismatches were incorporated at position m = 10 or n = 24, respectively. The intensity ratios are given in Table 9b. Fig. 13B is an autoradiogram of a capture reaction using 34–BrdC–D–E@10 under conditions leading to trace levels of capture.

FIGURE 13



A: Autoradiogram using same conditions as Fig. 11, except that D is 34–dC–D.

B: Autoradiogram at early time point using 34–BrdC–D–E@10.

C: Autoradiogram using same conditions as Fig. 13A.

TABLE 9: Linker Capture

a. Cases I and II:

	Displacer			
	34–BrdC–D		34–dC–D	
	Experimental	Calculated	Experimental	Calculated
Band:				
1807 nt	16	9	4	4
720 nt	384	344	16	15
308 nt	1*	1*	1*	1*

* by definition

b. Case III:

	Relative Yield (720nt/1807nt)	
Displacer	Experimental	Calculated
34–BrdC–D	24	39
34–BrdC–D–E@10	1	1
34–BrdC–D–E@24	24	39

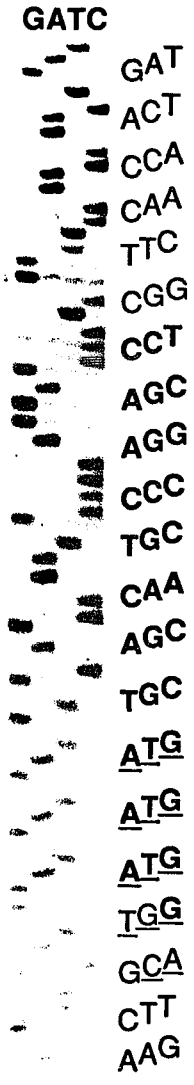
The capture by the 1807 nt band was exactly the same as that of the 720 nt band. A mismatch at position 10, for the 720 nt band, was equivalent to complete mismatching beginning at position 10, for the 1807 nt band. Thus, a single mismatch at position 10 blocked subsequent branch migration, and μ is very small and does not contribute to the partition function calculations.

Fig. 13C shows an autoradiogram analogous to Fig. 12A, where the displacer strand is 34–BrdC–D–E@24 instead of 34–BrdC–D. The results were similar to those Fig. 12A. Thus, the formation of the final singly–branched structure (Fig. 9, lower right) is unnecessary to achieve the high specificity observed for capture reactions of BrdC–containing oligodeoxynucleotides. This result requires $\Omega \geq 1$, which agrees with the calculations above.

3. CLONING AND SEQUENCING

The plasmid, pALA–D, was digested with SmaI and PstI, leading to three fragments of 2701, 1037, and 70 nt. Fragment 1037 had a PstI sites at each end. One of these sites contained the sequence complementary to 34–BrdC–D. The other fragments, including the pUC9 vector, terminated in one PstI site and one blunt SmaI site. The capture reaction (first ligation) was carried out as described above using 34–BrdC–D and 14–dC–L. The products were ligated into SphI and PstI digested pUC19. The SphI end contained a 3'–overhang complementary to the overhang created by the capture reaction. Four independent plasmid clones were digested by various restriction endonucleases and shown to have the patterns expected for linker incorporation. One of these clones was chosen for double–stranded DNA sequencing. The autoradiogram of the sequencing gel depicted in Fig. 14

FIGURE 14



Autoradiogram of sequencing gel showing the region of incorporated displacer (bold) and linker (underlined) sequences.

confirmed the capture reaction with incorporation of the linker sequence. These results demonstrated that a branched structure incorporating a BrdC–containing oligodeoxynucleotide could be cloned into E. coli.

E. DISCUSSION

We have confirmed that the substitution of bromodeoxycytidine for deoxycytidine increases DNA duplex stability and have demonstrated that displacement reactions using BrdC–substituted oligodeoxynucleotides are rapid, especially if initiated at a dangling end. In fact, when 4 complementary deoxynucleotides were added to both the BrdC–containing strand and the complementary strand in the duplex, the rate constant was 440 $M^{-1}sec^{-1}$ at 27°C and increased with decreasing temperature. This rate of displacement is of the same order of magnitude as DNA reassociation with nucleation limited to a 4–base region (Wetmur, 1976). These thermodynamic and kinetic measurements formed the basis of a system which permits sequence–dependent capture of displacer–linker (D–L) complexes.

Capture of a D–L duplex at the end of an R duplex can be carried to 100% completion by the addition of DNA ligase to the complexes. Our capture experiments were carried out using conditions where the half time to achieve equilibrium between D–L complexes and a recipient duplex end was about 1 minute for oligonucleotide concentrations of 6μg/ml for D and 2μg/ml for L. Thus, the rate–determining step was ligation. The results presented in this work are limited to R duplexes with 3' overhangs. We have found that BrdC–substituted D molecules were required to obtain a high specificity of Case II reactions compared to capture of D–L duplexes by ends with limited

complementarity to the D molecule. In addition, the introduction of a single mismatch in the D molecule was found to be sufficient to block the branch migration. We have demonstrated that the BrdC–containing, branched structure resulting from the capture reaction can be ligated into a cloning vector and propagated in E. coli. Thus, branch migration of a BrdC–substituted displacer oligodeoxynucleotide followed by ligation–mediated linker capture can be the basis of a selective cloning technique.

For the purposes of this demonstration, no attempt was made to remove the competing D–L duplexes prior to the second ligation.

There are two general applications for our capture system. Firstly, specific restriction fragments can be labeled to facilitate their detection following gel electrophoresis without the need for blotting and Southern hybridization. Fluorescent bases could be incorporated or the linker oligonucleotide could be end–labeled with ^{32}P using either T4 polynucleotide kinase, as described above, or terminal deoxynucleotide transferase if higher specific activity is desired (Church and Kieffer–Higgins, 1988). The linker could also contain biotinylated nucleotides, which could be used for purification of a particular fragment by affinity chromatography (Rigas, et al., 1986).

The second application of the capture system is branch migration–mediated DNA cloning. Linker capture at both ends of a particular fragment would increase the cloning selectivity to the product of the selectivity at each end (i.e. to 10,000 fold). The high selectivity would require the design of two sets of D and L molecules, one for each end of a fragment of interest. Capture at both ends is more important for cloning than for end–labeling, where selectivity could only be increased to the sum of the label captured at the two ends.

The work described in this communication was limited to the use of synthetic BrdC–containing oligodeoxynucleotides. Any other nucleotide which increased duplex stability and which did not interfere with DNA cloning could substitute for BrdC. However, at the present time, BrdC is the only useful nucleotide available as a phosphoramidite. Furthermore, BrdC may be incorporated into DNA enzymatically (Kornberg, 1980). For example, enzymatically–synthesized oligodeoxynucleotides could be used to simplify chromosome walking. A displacer strand could be produced by runoff replication (Saiki, et al., 1988) where only the 5' sequence prior to the 3'–overhang restriction site is known.

Our capture technology also could simplify repetitive cloning for the purpose of examining allele–specific gene polymorphisms or mutations in a diploid organism. The polymerase chain reaction (Saiki, et al., 1988) is a powerful tool for obtaining relatively short sequences of genes of interest. However, the assignment of such sequences to one or the other allele is problematic. The only practical method for obtaining the contiguous sequence of a particular allele of a eukaryotic, autosomal gene (e.g. 10–50 kb) would be to obtain allele–specific clones prior to sequencing. Branch migration–mediated DNA cloning may be carried out with cloning vectors that accept either small or large fragments of chromosomal DNAs, including plasmids, bacteriophages, cosmids and yeast artificial chromosome vectors.

V. DISCUSSION

A. METHYLPHOSPHONATE THERMODYNAMICS

Methylphosphonate–substituted oligodeoxynucleotides are one type of modified nucleic acid which has been employed for antisense studies (Smith, et al., 1986; Agris, et al., 1986; Marcus–Sekura, et al., 1987; Sarin, et al., 1988; Maher & Dolnick, 1988). The methylphosphonate internucleotide linkage is chiral and uncharged, and its presence in an oligodeoxynucleotide quantitatively changes the biophysical characteristics of the oligodeoxynucleotide. In chapter II, a fully methylphosphonate–substituted 12–mer oligodeoxynucleotide (10 methylphosphonate and 1 phosphodiester bond) as well as 14–mers containing 3 methylphosphonate and 10 phosphodiester bonds were used to examine the effect of the methylphosphonate linkage on the thermodynamics of the helix–coil transition. In these studies, methylphosphonate as well as analogous phosphodiester oligodeoxynucleotides were hybridized to complementary sequences which extended beyond the duplex region in both the 3' and 5' directions, as a model for hybridization to single–stranded DNA.

By carrying out melting analyses with 14–mer oligodeoxynucleotides containing methylphosphonate linkages that were non–adjacent and 3' to only one of the four deoxynucleotides, we found that there was no significant sequence specificity for helix destabilization due to these modified linkages. The concentration–dependent melting profiles of these 14–mers were examined spectrophotometrically as a function of ionic strength, using the same methods as have been used with phosphodiester oligodeoxynucleotides

(Marky, et al., 1983; Arnold, et al., 1987; Woodson & Crothers, 1987) as well as polydeoxynucleotides and DNAs (Wells, et al., 1970; Ornstein & Fresco, 1983; Marmur & Doty, 1962).

We made use of a gel migration assay to study duplex formation between a complementary ^{32}P–labeled phosphodiester and the fully methylphosphonate–substituted oligodeoxynucleotide because it consisted of a large number of diastereoisomers which would lead to duplexes with differing melting temperatures. The assay also allowed quantitation of the oligodeoxynucleotide by stoichiometry of binding to the labeled complement. Since the fully modified oligodeoxynucleotide was considerably lower in charge than its analogous phosphodiester, hybrids made between these sequences and the labeled complement had easily distinguishable electrophoretic mobilities. Thus, we were able to follow the competition between the modified and unmodified oligodeoxynucleotides for hybrid formation as a function of salt.

Analysis of thermodynamic data from various sources for oligodeoxynucleotides and polymeric and natural DNAs yielded nearest neighbor free energy values which can be used to accurately predict concentration–dependent melting temperatures for all phosphodiester oligodeoxynucleotides, including those containing adjacent GG nearest neighbors. The results also provided a basis for determining the free energy contribution of 3' and 5' dangling ends to helix stability (about 1 kcal/mol), and for the quantitative expression of the effect of methylphosphonate linkages at all ionic strengths on helix stability.

The free energy decrement due to introduction of each methylphosphonate linkage was found to be –0.75 kcal/mol in high salt. This

value represents an average of the detrimental effects of each of two isomeric forms (R and S), since the oligodeoxynucleotides studied contained mixtures of the two linkages. A mixture had to be employed because synthesis of multiply–substituted methylphosphonate oligodeoxynucleotides containing only one linkage form would have been unwieldy.

The equivalent hyperchromicities found for the methylphosphonate and phosphodiester 14–mer oligodeoxynucleotides indicated that all of the diastereoisomeric forms of the partially modified oligodeoxynucleotides (there are 8 for each) were capable of hybridizing with their complements. Using these oligodeoxynucleotides, we determined that the change in charge between the helix and coil forms, per nearest neighbor base pair, decreased from 0.26 for phosphodiester DNA, to 0.0 when the nearest–neighbor base pair contained one methylphosphonate. This result accounted for the fact that, in spite of the helix–destabilizing effect, methylphosphonate–substituted oligodeoxynucleotides formed more stable hybrids than analogous phosphodiester sequences at very low salt.

We demonstrated that, at low salt (below 10 mM), the fully–substituted methylphosphonate oligodeoxynucleotide also formed a more stable hybrid than the analogous phosphodiester sequence. However, a comparison of predicted values for t_m for 12–Me[10] and 12–P1, based on the experiments with the 14–mers, indicated that they should form hybrids of equal stability with 18–P1 at 2 mM. Our observation of a greater relative affinity of 12–Me[10] for 18–P3 suggested that the diastereoisomers of 12–Me[10] which take part in duplex formation may actually have smaller average detrimental $\Delta G°_d$ values than the –0.75 kcal/mol average value used for the calculations. Perhaps only the subset of the diastereoisomers composed predominantly of the R form of the

methylphosphonate linkage were actually capable of participating in duplex formation. Nevertheless, 12–Me[10] still maintained a high degree of specificity for its complementary sequence, because it specifically bound to the DNA fragment containing its complement in a Southern (1975) blot.

Finally, analysis of the values of the time–dependent dissociation temperatures for the partially substituted oligodeoxynucleotides, as determined by elution from dot blots in various salt concentrations, indicated that substitution of methylphosphonate linkages only affected the reverse rate constant.

If one contemplates the use of antisense oligodeoxynucleotides in cells, where the potassium ion concentration is approximately 140 mM, there would be no advantage in hybrid formation due to the use methylphosphonate–substituted oligodeoxynucleotides, but their increased stability to nucleases (chapter III) and lipophilic nature (Miller, et al., 1981) may still make them preferable to unmodified, phosphodiester oligodeoxynucleotides.

B. METHYLPHOSPHONATE BIOCHEMISTRY

In chapter III, we investigated some of the biochemical properties of methylphosphonate–substituted oligodeoxynucleotides which are pertinent to their use as antisense agents. A series of partially methylphosphonate–substituted oligodeoxynucleotides were compared to analogous phosphodiester oligodeoxynucleotides for sensitivities to specific nucleases in vitro, stability in tissue culture, and for their ability to promote cleavage of complementary RNA by RNase H. The different spatial arrangements of methylphosphonate and phosphodiester linkages in the modified oligodeoxynucleotides allowed us to

find general relationships between the various biochemical properties examined and overall oligodeoxynucleotide structure.

The gel migration assay from chapter II was used to asses the effects of nuclease or tissue culture treatment, where tested oligodeoxynucleotides were examined for their ability to form a duplex with a ^{32}P–labeled complementary oligodeoxynucleotide. The overall results of the nuclease and tissue culture studies indicated that partial methylphosphonate–substitution can be quite effective in prolonging the half–life of an oligodeoxynucleotide. A single methylphosphonate linkage was found to increase resistance to exonucleases by more than two orders of magnitude. The results of the studies of DNase I sensitivity suggest that endonuclease protection is maximized by minimizing the number of internal contiguous phosphodiester linkages. A fully–substituted methylphosphonate oligodeoxynucleotide would certainly be best protected, but in the interest of promoting stable duplex formation, internal spans of 3 phosphodiester linkages would be adequate (300 fold protection). In fact, because the tissue culture results demonstrated uniform half–lives for methylphosphonate oligodeoxynucleotides with internal contiguous phosphodiester linkages ranging from 1 to 4, a span as large as 4 may be sufficient in an antisense oligodeoxynucleotide for use in cell culture. We find that it is the degradative elements present in serum which are factors in oligodeoxynucleotide half–life.

Fully–substituted, antisense methylphosphonate oligodeoxynucleotides presumably function by creating a physical block to the protein translational machinery because the hybrids they form with RNA are not recognized as substrates by RNase H (Maher & Dolnick, 1988). The results of our RNase H assays show that partially methylphosphonate–substituted

oligodeoxynucleotides with 3 or more contiguous phosphodiester linkages will efficiently promote RNase H cleavage of complementary RNA. This is compatible with a highly nuclease–resistant arrangement of methylphosphonate and phosphodiester linkages.

C. BRANCH MIGRATION–MEDIATED LABELING AND CLONING

The studies in chapter IV led to the development of a technique for sequence–specific labeling and cloning of DNA. In this technique a BrdC–containing "displacer" oligodeoxynucleotide is used to invade the end of a specific fragment of DNA by branch migration. The displacer is then "captured" along with a partially complementary "linker" oligodeoxynucleotide by the action of DNA ligase.

Substitution of dC with BrdC in nucleic acids results in increased duplex stability (Inman & Baldwin, 1964), and polynucleotides containing BrdC displace their dC–containing analogues from pre–formed duplexes (Inman, 1964). In our preliminary studies we carried out comparative melting temperature analyses for BrdC–containing oligodeoxynucleotides and their dC–containing analogues. The T_m analyses, performed as a function of pH, confirmed the observation of increased duplex stability due to BrdC substitution. We derived the pH–dependent values for the average change in free energy ($\Delta G°_d$) which related to the increase in T_m. At pH 7.0, $\Delta G°_d = 0.4$ kcal/mol, so the relative stability of a BrdC–dG base pair versus a dC–dG base pair (K) is 1.9. This value was of importance in our calculations of the partition functions which described various reactions in our branch–migration mediated technique. We also investigated the displacement by BrdC–

substituted oligodeoxynucleotides of their dC–containing analogues from duplexes with blunt and 3'–overhanging ends. The gel migration assay used in the methylphosphonate studies was employed here to demonstrate these displacement reactions, where displacement of a ^{32}P–labeled dC–containing analogue was evidenced by its failure to gel shift. The displacement rates were temperature dependent. For the blunt–ended duplexes this may be due to increased breathing of the ends as the temperature is elevated. For duplexes with overhangs, the observed increase in rates with decreasing temperature is probably due to an increase in duplex stability between the region of the overhang and the BrdC–containing displacer.

The displacement reaction studies were the models for the specific capture reaction. We investigated the three cases of branch migration discussed in Theoretical of chapter IV, and found, both by calculation and experimentally, that the capture rate at the correct PstI site was over 300 times that at an unrelated PstI site, only when a BrdC displacer was used. A dC displacer afforded no such high specificity. Also of importance to the specificity of the technique was the demonstration that incorporation of a mismatched nucleotide in the displacer caused branch migration to be terminated. The use of BrdC–substitutions is compatible with the application of this technique to cloning, because a branched, BrdC–containing capture product was successfully cloned and sequenced.

VI. SIGNIFICANCE

Biophysical properties of methylphosphonate– and BrdC–substituted oligodeoxynucleotides have been investigated. The studies were undertaken to develop a better understanding of the characteristic behaviors of these modified nucleic acids as a basis for the improvement of existing technologies and for the development of new applications.

The thermodynamics of the helix–coil transition for methylphosphonate–substituted oligodeoxynucleotides has been described in terms of the decrement to the free energy and the nearest neighbor change in charge density due to the modified linkages. The decrease in duplex stability caused by methylphosphonate substitution is reflected by the effect on the reverse rate constant. Concomitant with these findings was the derivation of nearest neighbor free energy values applicable to the accurate prediction of melting temperatures for phosphodiester oligodeoxynucleotides, including those with many GG nearest neighbor interactions.

Results of nuclease sensitivity, tissue culture stability, and RNase H studies of partially methylphosphonate–substituted oligodeoxynucleotides suggest a general arrangement of phosphodiester and methylphosphonate linkages that should be compatible with efficient antisense activity in cultured cells and perhaps in vivo.

The effect of BrdC–substitution on oligodeoxynucleotide melting was defined in terms of the relative stability of a BrdC–dG base pair over a dC–dG base pair. Displacement of a dC–containing strand from a duplex by a BrdC–containing analogue was the basis of a new method of branch migration–mediated, site–specific DNA labeling and cloning method.

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