US005808045A

United States Patent [19]

Hiatt et al.

[11] **Patent Number: 5,808,045**

[45] **Date of Patent: *Sep. 15, 1998**

[54] **COMPOSITIONS FOR ENZYME CATALYZED TEMPLATE-INDEPENDENT CREATION OF PHOSPHODIESTER BONDS USING PROTECTED NUCLEOTIDES**

[75] Inventors: **Andrew C. Hiatt**, 660 Torrance St., San Diego, Calif. 92103; **Floyd Rose**, Del Mar, Calif.

[73] Assignees: **Andrew C. Hiatt**, San Diego; **Floyd D. Rose**, Del Mar, both of Calif.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,763,594.

[21] Appl. No.: **486,897**

[22] Filed: **Jun. 7, 1995**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 300,484, Sep. 2, 1994, abandoned.

[51] **Int. Cl.**[6] **C07H 19/04**

[52] **U.S. Cl.** **536/26.26**; 536/26.7; 536/26.71; 536/26.72; 536/26.74; 536/26.8

[58] **Field of Search** 536/26.26, 26.7, 536/26.71, 26.72, 26.74, 26.8

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,096,324	6/1978	Kelly et al. .
4,423,212	12/1983	Skulnick .
4,719,176	1/1988	Koltz .
4,820,812	4/1989	Miyoshi et al. .
4,863,849	9/1989	Melamede .
4,876,335	10/1989	Yamane et al. .
4,948,882	8/1990	Ruth .
4,950,745	8/1990	Ishido et al. .
4,980,460	12/1990	Molko et al. .
5,003,059	3/1991	Brennan .
5,032,680	7/1991	Kawai et al. .
5,039,796	8/1991	Engels et al. .
5,047,524	9/1991	Andrus et al. .
5,093,232	3/1992	Urdea et al. .
5,112,963	5/1992	Pielses et al. .
5,256,549	10/1993	Urdea et al. .
5,258,506	11/1993	Urdea et al. .
5,262,530	11/1993	Andrus et al. .
5,262,536	11/1993	Hobbs, Jr. .
5,264,563	11/1993	Huse .
5,264,566	11/1993	Froehler et al. .
5,268,266	12/1993	Fritsch et al. .
5,268,464	12/1993	Brill .
5,281,701	1/1994	Vinayak .
5,302,509	4/1994	Cheeseman .
5,348,868	9/1994	Reddy et al. .
5,362,866	11/1994	Arnold, Jr. .
5,367,066	11/1994	Ureda et al. .
5,380,833	1/1995	Urdea .
5,436,143	7/1995	Hymann et al. .

FOREIGN PATENT DOCUMENTS

55-38324	3/1980	Japan .

OTHER PUBLICATIONS

Bollum, *Fed Proc. Soc. Exp. Biol. Med.*, 17, 193 (1958).
Deng and Wu, *Meth. Enzymol.*, 100: 96–116 (1983).
Kaufmann et al., *Eur. J. Biochem*, 24:4–11 (1971).
Hinton and Gumport, *Nucleic Acid Res.*, 7:453–464 (1979).
Modak, *Biochemistry*, 17, 3116–3120 (1978).
England and Uhlenbeck, *Biochemistry*, vol. 17, 11:2069–2076 (1978).
Chang and Bollum, *Biochemistry*, vol. 10, 3:536–542 (1971).
Bennett et al., *Biochemistry*, vol. 12, 20:3956–3960 (1973).
Kossel and Roychoudrury, *Eur J. Biochem.*, 22:271–276 (1971).
Flugel et al., *Biochem. Biophys. Acta*, 308:35–40 (1973).
Sarfati et al., *J. Biol Chem.*, 265(31), 18902–18906 (1990).
Metzker et al., *Nucleic Acids Res.*, 22(20), 4259–4267 (1994).
Canard et al., *Proc. Nat'l. Acad. Sci. USA*, 92, 10859–10863 (Nov. 1995).
Canard et al., *Gene*, 148, 1–6 (1994).
Kutateladze et al., *FEBS*, 207(2), 205–212 (1986).

Primary Examiner—James O. Wilson
Attorney, Agent, or Firm—Lerner, David, Littenberg, Krumholz & Mentlik

[57] **ABSTRACT**

A method for the stepwise creation of phosphodiester bonds between desired nucleosides resulting in the synthesis of polynucleotides having a predetermined nucleotide sequence by preparing an initiation substrate containing a free and unmodified 3'-hydroxyl group; attaching a mononucleotide selected according to the order of the predetermined nucleotide sequence to the 3'-hydroxyl of the initiating substrate in a solution containing a catalytic amount of an enzyme capable of catalyzing the 5' to 3' phosphodiester linkage of the 5'-phosphate of the mononucleotide to the 3'-hydroxyl of the initiating substrate, wherein the mononucleotide contains a protected 3'-hydroxyl group, whereby the protected mononucleotide is covalently linked to the initiating substrate and further additions are hindered by the 3'-hydroxyl protecting group. Methods in which a mononucleotide immobilized on a solid support is added to a free polynucleotide chain are also disclosed.

7 Claims, No Drawings

COMPOSITIONS FOR ENZYME CATALYZED TEMPLATE-INDEPENDENT CREATION OF PHOSPHODIESTER BONDS USING PROTECTED NUCLEOTIDES

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. patent application Ser. No. 08/300,484 filed Sep. 2, 1994.

TECHNICAL FIELD

This invention relates to the synthesis of oligonucleotides and other nucleic acid polymers using template independent enzymes.

BACKGROUND OF THE INVENTION

Oligonucleotides are presently synthesized in vitro using organic synthesis methods. These methods include the phosphoramidite method described in Adams et al., *J. Amer. Chem. Soc.,* 105:661 (1983) and Froehler et al., *Tetrahedron Lett.,* 24:3171 (1983) and the phosphotriester method described in German Offenlegungsshrift 264432. Other organic synthesis methods include those described by Froehler et al., U.S. Pat. No. 5,264,566 in which H-phosphonates are used to produce oligonucleotides.

The phosphoramidite method of phosphodiester bond formation and oligonucleotide synthesis represents the current state of the art employed by most laboratories for the coupling of desired nucleotides without the use of a template. In this method, the coupling reaction is initiated by a nucleoside attached to a solid support. The 5'-hydroxyl group of the immobilized nucleoside is free for coupling with the second nucleoside of the chain to be assembled. Since the growing oligonucleotide chain projects a 5'-hydroxyl available for reaction with a mononucleotide, the direction of synthesis if referred to as 3' to 5'.

Each successive mononucleotide to be added to the growing oligonucleotide chain contains a 3'-phosphoramidate moiety which reacts with the 5'-hydroxyl group of the support-bound nucleotide to form a 5' to 3' internucleotide phosphodiester bond. The 5'-hydroxyl group of the incoming mononucleotide is protected, usually by a trityl group, in order to prevent the uncontrolled polymerization of the nucleosides. After each incoming nucleoside is added, the protected 5'-hydroxyl group is deprotected, so that it is available for reaction with the next incoming nucleoside having a 3'-phosphoramidite group and a protected 5'-hydroxyl. This is followed by deprotection and addition of the next incoming nucleotide, and so forth.

Between each nucleoside addition step, unreacted chains which fail to participate in phosphodiester bond formation with the desired nucleoside are chemically "capped" to prevent their further elongation. This usually involves chemical acetylation.

This method and the other currently used organic methods while widely accepted require large amounts of costly monomers that require complex organic synthesis schemes to produce. In addition, these methods are complex in that the phosphoramidite method requires an oxidation step after each condensation reaction. The phosphotriester method requires that the subpopulation of oligonucleotides that have not had a monomer added in a particular cycle be capped in a separate reaction to prevent further chain elongation of these oligonucleotides.

Other drawbacks of virtually all chemical methods of phosphodiester bond formation, is that the reaction must be performed in organic solvents and in the absence of water. Many of these organic solvents are toxic or otherwise hazardous. Another drawback to chemical synthesis is that it is at best 98 percent efficient at each cycle. In other words, following each nucleotide addition, at least 2 percent of the growing oligonucleotide chains are capped, resulting in a yield loss. The total yield loss for the nucleotide chain being synthesized thus increases with each nucleotide added to the sequence.

For example, assuming a yield of 98 percent per nucleotide addition, the synthesis of a polynucleotide consisting of 70 mononucleotides would experience a yield loss of nearly 75 percent. Furthermore, the object nucleotide chain would require isolation from a reaction mixture of polynucleotides, nearly 75 percent of which consist of capped oligonucleotides ranging between 1 and 69 nucleotides in length.

This inherent inefficiency in chemical synthesis of oligonucleotides ultimately limits the length of oligonucleotide that can be efficiently produced to oligonucleotides having 50 nucleic acid residues or less.

A need exists for a method which improves the efficiency of phosphodiester bond formation and which could ultimately be capable of producing shorter chain oligonucleotides in higher yields and longer chain polynucleotides in acceptable yields. In addition, a need exists for a polynucleotide synthesis system which is compatible with pre-existing polynucleotides, such as vector DNAs, so that desired polynucleotide sequences can readily be added on to the pre-existing sequences. Chemical coupling by the phosphoramidite method is not compatible with "add-on" synthesis to pre-existing polynucleotides. Enzyme catalyzed phosphodiester bond formation, however, can be performed in an aqueous environment utilizing either single or double stranded oligo- or polynucleotides to initiate the reaction. These reaction conditions also greatly minimize the use of toxic and hazardous materials.

The 3' to 5' direction of synthesis inherent to the phosphoramidite method of phosphodiester bond formation cannot be enzyme catalyzed. All known enzymes capable of catalyzing the formation of phosphodiester bonds do so in the 5' to 3' direction since the growing polynucleotide strand always projects a 3'-hydroxyl available for attachment of the next nucleoside.

There are many enzymes capable of catalyzing the formation of phosphodiester bonds. One class of such enzymes, the polymerases, are largely template dependent in that they add a complementary nucleotide to the 3' hydroxyl of the growing strand of a double stranded polynucleotide. However, some polymerases are template independent and primarily catalyze the formation of single stranded nucleotide polymers. Another class of enzyme, the ligases, are template independent and form a phosphodiester bond between two polynucleotides or between a polynucleotide and a mononucleotide.

Addition of single nucleotides to DNA fragments, catalyzed by deoxynucleotidyl terminal transferase (TdTase), has previously been described by Deng and Wu, *Meth. Enzymol.,* 100:96–116, 1983. These reaction conditions did not involve transient protection of the 3'-hydroxyl nor were they intended to be used for the sequential creation of phosphodiester bonds to synthesize a predetermined nucleotide sequence. The presence of unprotected 3'-hydroxyls resulted in a highly heterogeneous population of reaction products.

Similarly, prior attempts to catalyze synthesis of very short pieces of RNA or DNA using protected nucleotide

monophosphates or diphosphates resulted in unacceptably low levels of the desired phosphodiester bond formation or required excessive amounts of enzyme to achieve acceptable efficiencies. These problems were largely due to unavoidable heterogeneity of the mononucleotide building blocks or to the very high turnover number of the enzyme, necessitating extremely long incubation times (see, for example, Hinton and Gumport, *Nucleic Acids Res.* 7:453–464, 1979; Kaufman et al., *Eur. J. Biochem.,* 24: 4–11, 1971). These experiments were limited to 5'-monophosphates and diphosphates. No attempts have been made to catalyze controlled DNA synthesis using 5'-triphosphates protected at the 3' position.

Enzyme catalyzed creation of a single phosphodiester bond between the free 3'-hydroxyl group of an oligonucleotide chain and the 5'-phosphate of a mononucleotide requires protection of the 3'-hydroxyl of the mononucleotide in order to prevent multiple phosphodiester bond formations and hence repeated mononucleotide additions. Protection of the 3'-hydroxyl of the mononucleotide ideally involves a transient blocking group which can readily be removed in order to allow subsequent reactions. Flugel et al., *Biochem. Biophys. Acta.* 308:35–40, 1973, report that nucleoside triphosphates with blocked 3'-hydroxyl groups cannot be prepared directly. This lack of 3' blocked triphosphates necessitated previous processes to utilize lower energy and thus more inefficient 3' blocked monophosphates and diposphates. Synthetic techniques to create 3' block triphosphates would be highly desirable, because this could enable stepwise enzyme catalyzed phosphodiester bond formation leading to polynucleotide synthesis.

These prior attempts at synthesizing oligonucleotides using a template independent polymerase were extremely inefficient resulting in the production of very short oligonucleotides. The inefficiency of these methods made these methods useless for practical synthesis of oligonucleotides.

The present invention allows the creation of phosphodiester bonds between nucleotides using a template independent polymerase to create oligonucleotides having a predetermined sequence. This enzyme catalysis can vastly improve the efficiency of phosphodiester bond formation between desired nucleotides compared to current techniques of chemical coupling and can be carried out in the presence of other biological molecules such as pre-existing sequences of single or double stranded DNA as well as other types of enzymes. In addition, the very high specificity inherent to enzyme catalysis allows only coupling of a 5'-phosphate to a 3'-hydroxyl. The coupling of two mononucleosides, as well as various other side reactions inherent to chemical coupling techniques, simply do not occur.

A further advantage of the present invention is realized by using 3' blocked triphosphates having high energy phosphate bonds which an enzyme can utilize to drive the reaction to greater completion level than when other monophosphates and diphosphates are used. In addition, triphosphates are less strongly hydrated than the diphosphate, which also tends to drive catalytic hydrolysis of the triphosphate to completion.

Clearly, the availability of a homogeneous population of protected mononucleotide triphosphates and enzymes capable of efficiently joining protected nucleotides to initiating substrates will enable the creation of a highly uniform population of synthetic polynucleotides resulting from stepwise phosphodiester bond formation.

SUMMARY OF THE INVENTION

A number of methods have been discovered by which the 3'-hydroxyl group of a deoxynucleotide triphosphate can be effectively protected and deprotected and wherein the protected nucleotide is utilized by a template independent polymerase to create a phosphodiester bond permitting the synthesis of oligonucleotides or polynucleotides having a desired predetermined sequence.

Therefore, in accordance with the present invention, a method is provided for the synthesis of a polynucleotide of a predetermined sequence of which method includes the steps of:

A. providing an initiating substrate comprising a nucleoside having an unprotected 3'-hydroxyl group; and

B. reacting under enzymatic conditions in the presence of a catalytic amount of an enzyme the 3'-hydroxyl group of the initiating substrate with a nucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position of the nucleoside 5'-triphosphate and selected according to the order of the predetermined sequence, so that enzyme catalyzes the formation of a 5' to 3' phosphodiester linkage between the unprotected 3'-hydroxyl group of the initiating substrate and the 5'-phosphate of the nucleoside 5'-triphosphate to produce the polynucleotide.

In other embodiments of the present invention, the method further comprises the step:

C. removing the blocking moiety protecting the 3' position of said nucleotide 5'-triphosphate to produce an initiating substrate having an unprotected 3'-hydroxyl group.

In other embodiments, steps (b) and (c) are repeated at least once to add additional nucleotides to the initiating substrate by alternatively adding a nucleoside 5'-triphosphate with a removable blocking moiety at its 3' position, deblocking the 3' position of the terminal nucleoside and then adding another nucleoside 5'-triphosphate with a removable blocking group at its 3' position. Repetition of steps (b) and (c) can also be carried out to produce an oligonucleotide or polynucleotide having a predetermined sequence.

The present invention contemplates initiating substrates that are deoxynucleosides, nucleotides, single or double stranded oligonucleotides, single or double stranded polynucleotides, oligonucleotides attached to nonnucleoside molecules and the like.

The present invention contemplates embodiments in which the substrate is immobilized on a solid support. Preferred solid supports include cellulose, Sepharose, controlled-pore glass, silica, Fractosil, polystyrene, styrene divinyl benzene, agarose, and crosslinked agarose and the like.

The present invention contemplates the use of template independent polynucleotide polymerases such as terminal deoxynucleotidyl transferase from any number of sources including eukaryotes and protharyotes.

The methods of the present invention utilize removable blocking moieties that block the 3' position of nucleoside 5'-triphosphates used in the methods. Preferred removable blocking moieties can be removed in under 10 minutes to produce a hydroxyl group at the 3' position of the 3' nucleoside. Removable blocking groups contemplated include carbonitriles, phosphates, carbonates, carbamates, esters, ethers, borates, nitrates, sugars, phosphoramidates, phenylsulfenates, sulfates and sulfones.

The methods of the present invention contemplate removing the removable blocking moiety using a deblocking solution that preferably contains divalent cations such as Co++ and a biological buffer such as comprises a buffer selected from the group consisting of dimethylarsinic acid,

tris[hydroxymethyl] amino methane, and 3-[m-morpholine] propanosulphonic acid. Other embodiments of the present invention utilize an enzyme present in the deblocking solution to remove the removable blocking moiety.

The present invention also contemplates methods in which the nucleoside 5'-triphosphate having the removable blocking moiety at its 3' position is immobilized in a solid support and reacted with free initiating substrates. The solid support is linked to the nucleoside 5'-triphosphate at the 3'-hydroxyl group, thereby acting as a removable blocking moiety at the 3' position. Attachment of the nucleoside to the support is transient, thereby enabling the release of the newly synthesized product from the support and regeneration of the free and unmodified 3'-hydroxyl to allow the next nucleotide addition to occur.

Thus, in some embodiments of the present invention the deblocking solution would remove the removable blocking moiety at the position of the nucleoside and thus release the growing polynucleotide from the solid support.

The present invention also includes polynucleotides having a predetermined sequence provided according to the methods of this invention. Applications for using polynucleotides and oligonucleotides of the present invention in molecular cloning and/or expression of genes, peptides or proteins.

Also contemplated by the present invention are compositions of matter comprising a catalytic amount of a template independent enzyme and a nucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position of said nucleoside 5'-triphosphate. Additional compositions of matter further comprising an initiating substrate are also contemplated.

BRIEF DESCRIPTION OF THE INVENTION

A. Definitions

DNA: Deoxyribonucleic acid.

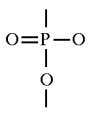
RNA: Ribonucleic acid.

Nucleotide: A subunit of a nucleic acid comprising a phosphate group, a 5-carbon sugar and nitrogen containing base. In RNA, the 5-carbon sugar is ribose. In DNA, it is a 2-deoxyribose. The term also includes analogs of such subunits.

Nucleoside: Includes a nucleosidyl unit and is used interchangeably therewith, and refers to a subunit of a nucleic acid which comprises a 5-carbon sugar and a nitrogen containing base. The term includes not only those nucleosidyl units having A, G, C, T and U as their bases, but also analogs and modified forms of the naturally-occurring bases, such as pseudoisocytosine and pseudouracil and other modified bases (such as 8-substituted purines). In RNA, the 5-carbon sugar is ribose; in DNA, it is 2'-deoxyribose. The term nucleoside also includes other analogs of such subunits, including those which have modified sugars such as 2'-O-alkyl ribose.

Polynucleotide: A nucleotide multimer generally about 50 nucleotides or more in length. These are usually of biological origin or are obtained by enzymatic means.

Phosphodiester: The group



wherein phosphodiester groups may be used as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

Hydrocarbyl: An organic radical composed of carbon and hydrogen which may be aliphatic (including alkyl, alkenyl, and alkynyl groups and groups which have a mixture of saturated and unsaturated bonds), alicyclic (carbocyclic), aryl (aromatic) or combination thereof; and may refer to straight-chained, branched-chain, or cyclic structures or to radicals having a combination thereof, as well as to radicals substituted with halogen atom(s) or heteroatoms, such as nitrogen, oxygen, and sulfur and their functional groups (such as amino, alkoxy, aryloxy, lactone groups and the like), which are commonly found in organic compounds and radicals.

Non-nucleoside monomeric unit: A monomeric unit wherein the base, the sugar and/or the phosphorus backbone or other internuclosidyl linkage of a nucleoside has been replaced by other chemical moieties.

Polypeptide and Peptide: A linear series of amino acid residues connected on to the other by peptide bonds between the alpha-amino and carboxyl groups of adjacent residues.

Protein: A linear series of greater than about 50 amino acid residues connected one to the other as in a polypeptide.

Gene: A segment of DNA coding for an RNA transcript that is itself a structural RNA, such as ribosomal RNA or codes for a polypeptide. The segment of DNA is also equipped with a suitable promoter, termination sequence and optionally other regulatory DNA sequences.

Structural Gene: A gene coding for a structural RNA and being equipped with a suitable promoter, termination sequence and optionally other regulatory DNA sequences.

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

Oligonucleotide: A chain of nucleosides which are linked by internucleoside linkages which is generally from about 2 to about 50 nucleosides in length. They may be chemically synthesized from nucleoside monomers or produced by enzymatic means. The term oligonucleotide refers to a chain of nucleosides which have internucleosidyl linkages linking the nucleoside monomer and, thus, includes oligonucleotide containing nucleoside analogs, oligonucleotide having internucleosidyl linkages such that one or more of the phosphorous group linkages between monomeric units has been replaced by a non-phosphorous linkage such as a formacetal linkage, a thioformacetal linkage, a sulfamate linkage, or a carbamate linkage. It also includes nucleoside/non-nucleoside polymers wherein both the sugar and the phosphorous moiety have been replaced or modified such as mopholino base analogs, or polyamide base analogs. It also includes nucleoside/non-nucleoside polymers wherein the vase, the sugar, and the phosphate backbone of a nucleoside are either replaced by a non-nucleoside moiety or wherein a non-nucleoside moiety is inserted into the nucleoside/non-nucleoside polymer. Thus an oligonucleotide may be partially or entirely phophonothioates, phosphorothioate phosphorodithioate phosphoramidate or neutral phosphate ester such as phosphotriesters oligonucleotide analogs.

Removable Blocking Moiety: A removable blocking moiety is a moiety which is attached to the oxygen at the 3' position of a nucleoside or the equivalent position in a nucleoside analog. The removable blocking moiety prevents reaction of the 3' oxygen when present and is removable under deblocking conditions so that the 3' oxygen can then participate in a chemical reaction.

A. Methods

Generally, the present invention provides methods for synthesizing oligonucleotides and polynucleotides having a predetermined sequence using a template independent polymerase and nucleoside having the 3' position blocked with a removable blocking moiety so that single nucleosides are added to the growing oligonucleotide. Single nucleosides are added to the growing chain by removing the blocking moiety at the 3' position of the terminal nucleoside of the growing oligonucleotide so that the next blocked nucleoside can be added to the oligonucleotide. This process is then repeated until the oligonucleotide having the predetermined sequence is produced.

Thus, in accordance with this embodiment of the present invention, a method comprises the steps of:

(a) providing an initiating substrate comprising a nucleoside having an unprotected 3'-hydroxyl group; and

(b) reacting under enzymatic conditions in the presence of a catalytic amount of an enzyme said 3'-hydroxyl group of said initiating substrate with a nucleoside 5'-triphosphate having a removable blocking moiety protecting the 3' position of said nucleoside 5'-triphosphate and selected according to the order of said predetermined sequence, whereby said enzyme catalyzes the formation of a 5' to 3' phophodiester linkage between said unprotected 3'-hydroxyl group of said initiating substrate and the 5'-phosphate of said nucleoside 5'-triphosphate to produce said polynucleotide.

In preferred embodiments, the methods of the present invention further comprises the step of:

(c) removing the blocking moiety protecting the 3' position of said nucleoside 5'-triphosphate to produce an initiating substrate having an unprotected 3'-hydroxyl group.

This additional step regenerates a reactive atom at the 3' position of the terminal nucleoside so that this atom can be used to form a bond with the next nucleoside and thus extend the length of the oligonucleotide by one nucleoside.

The methods of the present invention also include methods in which the above steps (b) and (c) are repeated at least once to produce an oligonucleotide. This process can be repeated many times to produce oligonucleotides of selected length. This process can also be repeated many times such that each particular nucleoside added to the oligonucleotide having a preselected sequence.

1. Initiating Substrates

An initiating substrate of the present invention is prepared containing a nucleoside with a free and unmodified 3'-hydroxyl group. As is well understood by those of ordinary skill in the art, nucleotide derivatives of the nucleosides adenosine, cytidine, guanosine, uridine and thymidine can be assembled to form oligonucleotides and polynucleotides. According to the method of the present invention, the initiating substrate may contain a single nucleoside having a free and unmodified 3'-hydroxyl group, or a preassembled oligo- or polynucleotide may be provided as an initiating substrate, so long as the oligo- or polynucleotide has a free and unmodified 3'-hydroxyl group.

One skilled in the art will understand that an initiating substrate could be provided in a form in which a nucleoside has a removable blocking moiety at its 3' position which is subsequently removed using a deblocking process so that the initiating substrate now has the free unprotected 3' hydroxyl group useful in the present invention.

The initiating substrates of the present invention include the termini of polynucleotides frequently generated and used in various cloning and molecular biology techniques. Examples of these initiating substrates include the termini of DNA or RNA vectors, single-stranded or double-stranded fragments, single-stranded or double-stranded RNA fragments and RNA or DNA oligonucleotides.

In the preferred embodiments, initiating substrates will consist wholly or in part of an oligo- or polynucleotide. The initiating substrate can be any arrangement of nucleosides which enables the enzyme to create a phosphodiester bond between the 3'-hydroxyl of a nucleoside and the 5'-phosphate of a mononucleotide. Initiating substrates may be based wholly or in part on ribonucleic acids (RNA) or deoxyribonucleic acids (DNA) and may be single stranded or multi-stranded. In addition, initiating substrates can include other types of naturally occurring or synthetic molecules (non-nucleosides) which may enable or enhance the ability of the enzyme to create a phosphodiester bond or which may facilitate the manipulation of reaction components and by-products. An example of this would be a linker molecule (commonly used linkers consist of C, O, N, and H e.g. Affi-Gel™ 10: R—$OCH_2CONH(CH_2)_2NHCO(CH_2)_2COON(CH_2)_2$ which would serve to provide a convenient method for attaching an initiating substrate to a solid support.

The sequential creation of phosphodiester bonds and hence the addition of nucleotides to the initiating substrate may be performed entirely in solution, or the initiating substrate may be attached to an insoluble matrix. Attachment to an insoluble matrix will permit the rapid separation of the substrate from unreacted reagents in order to prepare the substrate for the addition of the next nucleotide. For this reason, the substrate is preferably affixed to a solid support matrix during each reaction creating a phosphodiester bond.

Insoluble matrices suitable for use as solid supports include cellulose, Sepharose™, controlled-pore glass (CPG), polystyrene, silica, agarose, and the like.

Reagents, buffers and solvents suitable for use with the present invention are capable of flowing through the solid support matrix, by which means the initiating substrate is brought into contact with these materials. The growing nucleotide chain remains attached to the solid support as the various reagents, buffers and solvents sequentially flow therethrough. The solid support matrix is preferably contained within a synthesis column, to which reagents, buffers and solvents are provided.

Attachment of the initiating substrate to the solid support can be by covalent bonding. Numerous methods for the covalent attachment of molecules to insoluble matrices have been described and are well understood by those of ordinary skill in the art. In the preferred embodiment an oligonucleotide chain may be linked to alkylamine derivatized polystyrene or CPG by way of a covalent phosphoramidate bond although numerous strategies for linking oligonucleotides to solid supports have been described. The choice of an appropriate linking strategy will depend on the specific requirements of stability, charge interactions, solubility and the like.

Alternatively, attachment of the initiating substrate to the solid support can be by non-covalent interactions. Numerous methods for the transient attachment of molecules to insoluble matrices have been described and are well understood by those of ordinary skill in the art. For example, an oligonucleotide derivative containing single or multiple biotin molecules may be attached to avidin-agarose or streptavidin-agarose to form a non-covalent linkage between the oligonucleotide and the insoluble agarose matrix.

In general, it is envisioned that single and double stranded oligo- and polynucleotides based on DNA or RNA may be

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.