Solid-Phase Supports for Oligonucleotide Synthesis

INTRODUCTION TO SOLID-PHASE SYNTHESIS

The quest to understand and create biological molecules has long challenged synthetic chemists. In particular, the chemical synthesis of peptides and nucleic acids has always been a major pursuit. The primary structure of these molecules is a linear assembly of repeating units linked together in a defined orientation. Although solution-phase synthetic methods for coupling small units together were developed many years ago, the large number of couplings needed to assemble useful sequences was daunting. This was because each step required some type of workup, extraction, or purification, and the labor and cumulative loss of material from all the manipulations rapidly became significant problems. Indeed, the pioneering work by Khorana (1979) on total gene synthesis was not considered of practical importance by some researchers because of the enormous effort involved.

The problems involved in performing so many repetitive steps were addressed by Merrifield (1965) with the introduction of solid-phase synthesis (Fig. 3.1.1). In this strategy, a large insoluble support is covalently linked to the end of the sequence being assembled. The product on the surface of the support is available to react with reagents in the surrounding solution phase. The extended products remain covalently linked to the insoluble support while unreacted reagents remain free in solution. Therefore, at the completion of each step, the products can be rapidly and conveniently isolated by simply washing the unbound reagents away from the support. This can be performed as easily as filtering off the support and washing it with solvent. The support with its attached product is then ready for immediate use in the next step, as long as moisture contamination has not been introduced (in which case the support must be dried before use). In practice, it is convenient to handle the supports inside sealed reactors or columns so exposure to the atmosphere is minimized. This is also ideal for automation and the necessary reagent additions and solvent washes are readily mechanized. The process of adding each unit is repeated over and over until the desired sequence has been assembled on the surface of the support. The product can then be released from the support by cleavage of the covalent attachment (linker arm), and after removing the protecting groups, the synthesis is complete.

This strategy was originally applied to peptide synthesis, but it is also applicable to other linear macromolecules, such as DNA and RNA (Beaucage and Iyer, 1992) and oligosaccharides (Adinolfi et al., 1996). Recently, there has been a great deal of interest in applying this strategy to the combinatorial synthesis of small molecules and a new field of solid-phase organic chemistry (SPOC) is rapidly developing (Fruchtel and Jung, 1996; Porco et al., 1997). In this review, the main focus is on supports for oligodeoxyribonucleotide and oligoribonucleotide synthesis. The synthetic strategies are often similar, particularly when synthetic libraries are prepared.

ADVANTAGES OF SOLID-PHASE SYNTHESIS

The principal advantage of solid-phase synthesis is the ease with which immobilized products can be separated from other reactants and by-products. The simple filtration and washing steps are readily automated, and the method is ideal for the synthesis of linear molecules, which require the repetition of the same steps for every chain extension cycle. The use of insoluble solid-phase supports also permits relatively small quantities of material to be synthesized, because the additional physical bulk of the support, which is ~10 to 100 times the mass of the attached nucleoside, can be handled more easily than the nucleoside alone. Also confinement of the support inside a synthesis column eliminates handling losses. A small synthesis scale is important because of the high cost of reagents. Very little material is required for many biochemical applications and most syntheses actually prepare much more material than required. Therefore, as instrumentation has improved, the synthesis scale has decreased. Presently, synthesis on a 40-nmol scale, instead of a 0.2- to 1-μmol scale, is preferred for may applications. Oligonucleotide synthesis on a picomole scale or less may eventually become more common (Weiler and Hoheisel, 1997). It is already possible to synthesize molecules on single beads and to characterize the picomole quantities of synthetic peptides (Rapp, 1997) or oligonu-

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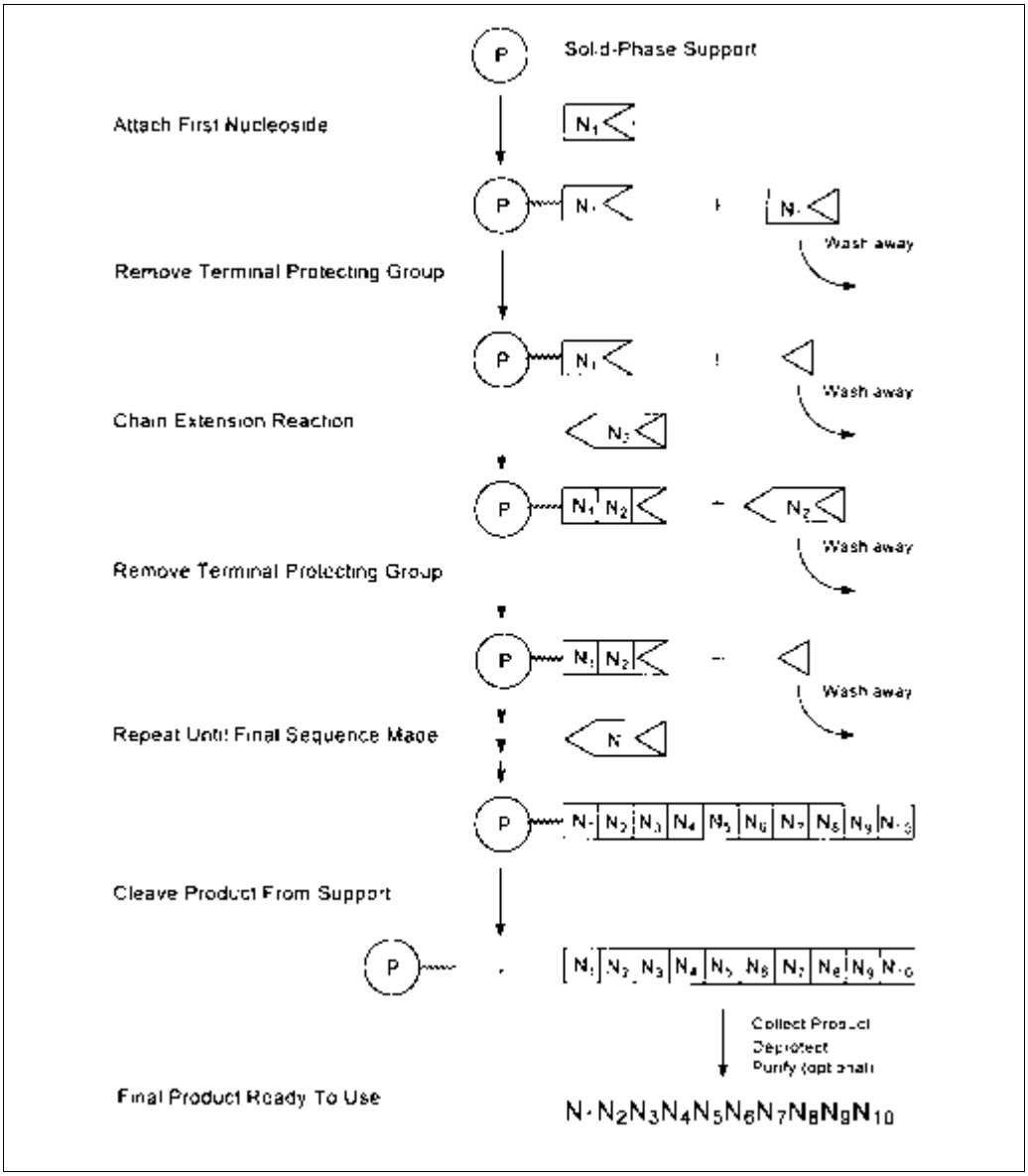

Figure 3.1.1 The general strategy for solid-phase oligonucleotide synthesis. The first step is attachment of a mononucleoside/tide (N_1) to the surface of an insoluble support (P) through a covalent bond. Excess monomers, which are not chemically attached to the support, are washed away. Before chain elongation can proceed, the terminal-protecting group (◁) on the nucleoside must be removed. This exposes a free 5′-OH or 3′-OH group, depending on the orientation of the synthesis. Usually synthesis proceeds from the 3′- to 5′-direction and the terminal protecting group is an acid-labile DMTr group. The next nucleotide unit (N_2) can then be added using the appropriate synthesis chemistry (usually phosphoramidite). An excess of reagent is used to force the coupling reaction to occur on as many of the immobilized nucleotides as possible. After the coupling reaction, excess reagents are washed away. Depending on the coupling chemistry, the reaction is followed by a capping step, to block off nonextended sites, and an oxidation step (these steps are not shown; see *UNIT 3.3* for details) to complete the chain-extension cycle. The process of terminal-protecting group removal and chain extension is then repeated, using different bases, until the desired sequence has been assembled. Some or all of the protecting groups may optionally be removed, and then the covalent attachment to the support is hydrolyzed to release the product. After removal of any remaining protecting groups, the oligonucleotide is ready for purification and use.

cleotides (Seliger et al., 1997) present on single beads.

The simplicity and similarity of the steps required for each chain extension reaction also greatly facilitate synthesis of modified oligonucleotides. As long as the modified substituents do not require any incompatible chemical treatments (i.e., to remove protecting groups), the inclusion of different bases and nucleosides, linkage inversions, branch points, non-nucleotide units, and end modifications can be readily accomplished. This is particularly so when the modified substituents are available as phosphoramidite derivatives, which use the same coupling chemistry as do regular bases (Beaucage and Iyer, 1993). Chimeric oligonucleotides containing peptide or peptide nucleic acid (PNA) sequences can, however, also be prepared (Bergmann and Bannwarth, 1995; Hyrup and Nielsen, 1996; van der Laan et al., 1997). Although, in these cases, the different coupling conditions and protecting groups require much more attention to ensure overall compatibility.

Finally, combinatorial methods can be used to create large numbers of different sequences. In the simplest application, multiple bases ("mixed bases") can be incorporated at defined positions by using a mixture of different monomers, instead of a single monomer, in the chain extension reaction. This procedure was originally developed to prepare oligonucleotide probes from peptide sequences when the exact codon usage was unknown. Later, this method became important when large libraries of degenerate or random sequences were required for in vitro selection experiments, such as the systematic evolution of ligands by exponential enrichment (SELEX) technique (Gold et al., 1995; see Chapter 9). Although DNA synthesizers can prepare mixed-base sites by on-line mixing, large numbers of degenerate sites are best made up by manually preparing solutions containing the desired ratio of nucleotides and incorporating the premixed reagents on the synthesizer. This is also the procedure used in base doping, when only one base, at random, within a particular section needs to be mutated (Hermes et al., 1989).

Another combinatorial approach was developed to simplify the synthesis of large numbers of oligonucleotides. This procedure used cellulose disks of filter paper as the insoluble support and became known as filter disk or segmented solid-phase synthesis (Frank et al., 1983; Matthes et al., 1984; Ott and Eckstein, 1984; Frank, 1993). In this procedure, multiple filter disks (each producing one unique oligonucleotide) are stacked together and handled at once. Reagents can be easily passed through the stack from top to bottom, and the number of oligonucleotides synthesized is limited only by the maximum stack height that can be manipulated. A different oligonucleotide sequence is prepared on each disk by interrupting the synthesis after each chain extension step. The individual filter disks are then sorted into separate piles according to the next base to be added. The insoluble support in this case provides the means to sort the products and to separate them from the excess reagents. For normal oligonucleotides, the sorting results in a maximum of four piles, because only dA, dC, dG, and T base additions are required. Thus an operator manipulating four concurrent syntheses can produce a large number of oligonucleotides per day. This method is not limited to paper filter disks; stackable "synthesis wafers" containing packets of support in bead form have also been used. The sorting step, however, is quite difficult to automate; and although semimechanized instruments have been reported (Seliger et al., 1987; Beattie et al., 1988), the segmented approach has not been widely adopted.

The ease with which immobilized oligonucleotides can be manipulated has also lead to the development of combinatorial strategies for the synthesis of oligonucleotide libraries. Unlike the above strategies, which release the oligonucleotide product from the support at the end of the synthesis, the oligonucleotides are left attached to the insoluble support (Markiewicz et al., 1994). This method can be used to create dispersed libraries, when the sequences are prepared on separate beads, or integrated libraries, when one- or two-dimensional arrays of sequences are prepared on a single surface. The sequence identity of each element in an integrated array is known from its spatial coordinates, whereas the sequence of elements in a dispersed library must be deduced from either direct sequencing (Seliger et al., 1997) or other sequence tags. The most elegant and powerful demonstration of this technique is the synthesis of high-density arrays on small (1.28 cm^2) glass chips using photolithography and light-sensitive protecting groups (Fodor et al., 1991). With the appropriate masking, any set of oligonucleotides of length N can be performed using only $4N$ coupling steps, and this technique can produce arrays of $>10^6$ different sequences (Lipshutz et al., 1995; McGall et al., 1996). Other combinatorial strategies using either glass plates (Milner et al., 1997) or

polypropylene sheets (Matson et al., 1995; Weiler and Hoheisel, 1996) as the insoluble support have been described for the synthesis of oligonucleotide arrays, although the array densities were much lower.

DISADVANTAGES OF SOLID-PHASE SUPPORTS

Although a powerful technique, solid-phase synthesis has some drawbacks. The main limitation is the need for very high coupling yields in every chain extension step. This is because the overall yield of product decreases rapidly as the number of consecutive chain extension steps increases (Fig. 3.1.2). For example, if each base addition step had a yield of 90%, then the amount of dinucleotide produced (one base addition) is 90%. The yield of trinucleotide (two base additions) is $0.90 \times 0.90 \times 100\% = 81\%$; the yield of tetranucleotide (three base additions) is $0.90 \times 0.90 \times 0.90 \times 100\% = 73\%$; and so on. Note that the first nucleoside is attached to the insoluble support before the start of oligonucleotide synthesis and the efficiency of that step is not included in the calculation. The mathematical relationship between the overall yield (*OY*) and the average coupling efficiency (*AY*) is either

$$OY = \left(\frac{AY}{100}\right)^{n} \times 100\%$$

or

$$OY = \left(\frac{AY}{100}\right)^{N-1} \times 100\%$$

where *n* is the number of coupling steps and *N* is the length of the oligonucleotide. The second equation assumes that the synthesis was performed by extending the product by one base at a time, as is usual.

The consequence of the exponential relationship between overall yield and average coupling efficiency is that long oligonucleotides cannot be prepared without very high yields in every step. The most difficult step is usually the coupling reaction; but in some strategies (e.g., light-directed synthesis of arrays or the use of liquid-phase supports), quantitative removal of the terminal-protecting group is also problematic. Coupling yields that would be acceptable for most solution-phase reactions (e.g., the 90% yield assumed in the above example) are not adequate; only yields >98% are acceptable. The lack of a coupling reaction that could reliably produce such high efficiencies was the major reason why solid-phase oligonucleotide synthesis was not successful until the early 1980s. After the discovery of trivalent phosphite–coupling chemistry and phosphoramidite derivatives (Caruthers, 1991), however, average coupling efficiencies of 99% or more were possible. Such high coupling efficiencies now allow oligonucleotides as long as 200 bases to be prepared (Bader et al., 1997b).

Another consequence of producing less than 100% coupling efficiencies is the accumulation of failure sequences containing deletions. The number of these failure products can be greatly reduced by the addition of a capping step after each chain extension reaction. This step, which typically uses acetic anhydride to acetylate nonextended molecules, prevents the failure

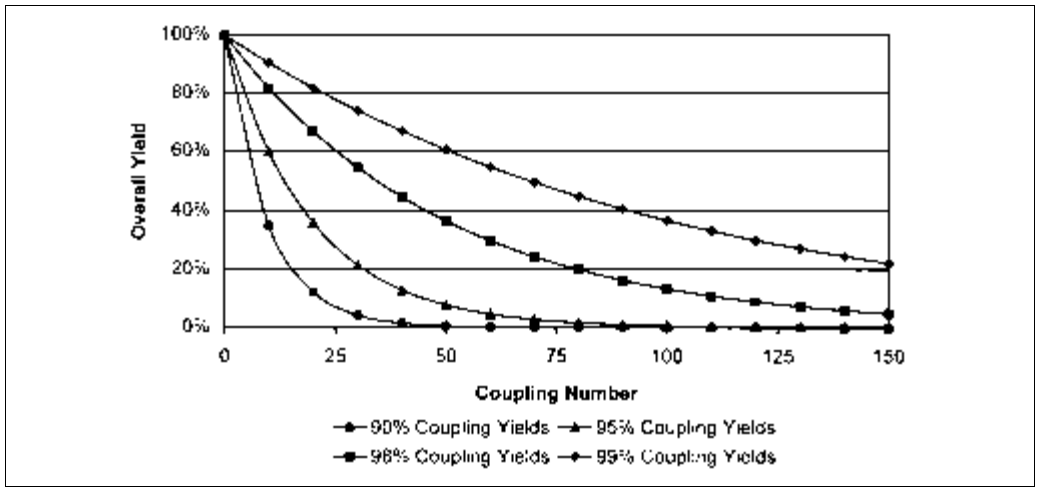

Figure 3.1.2 Overall yield vs. number of couplings. The overall yield of full-length product decreases with the number of coupling reactions for average coupling efficiencies of 90%, 95%, 98%, and 99%.

sequences from participating in any further reactions; however, a series of failure sequences, each one base shorter than the desired full-length product, will be present at the end of the synthesis.

Separating the full-length product (of length *N*) from the shorter failure sequences and especially the $N - 1$ failure sequence is another significant problem. This purification step becomes more difficult as oligonucleotide length increases, and for oligonucleotides greater than ~30 bases long, only polyacrylamide gel electrophoresis (PAGE) has sufficient resolving power to separate the full-length product from the $N - 1$ component. Fortunately, however, many biochemical applications do not have stringent purity requirements; and if the coupling efficiency was high enough, the mixture of products produced can often be used with either minimal (desalting) or no purification (Pon et al., 1996).

Analysis of the synthetic products still attached to the surface of the insoluble support also presents a major difficulty for researchers developing new techniques or new solid-phase supports. This is an especially significant problem for applications using immobilized arrays, because removal of the products for characterization is often difficult, if not impossible. Nuclear magnetic resonance (NMR) studies of immobilized products on solvent-swollen (gel-phase) polymers (Bardella et al., 1993) can be performed; but because such supports are not preferred for oligonucleotide synthesis, there have been few studies relating to oligonucleotide synthesis. Rigid supports can be studied using NMR and magic angle spinning, but there has been only one report of ^{31}P NMR performed on controlled-pore glass (CPG) particles with oligonucleotides (Macdonald et al., 1996). Recently, ellipsometry, interferometry, and optical wave guides have been used to study oligonucleotide arrays (Stimpson et al., 1995; Gray et al., 1997), but these techniques do not provide specific information about the fidelity of the oligonucleotide synthesis.

Finally, the cost of the support is a major factor when performing large-scale syntheses because, even with high loading supports, ~3 g of support is required for each gram of oligonucleotide product. Because most supports are expensive and can be used only once, there is a strong economic incentive to develop methods for regenerating and reusing the supports, especially when tonne quantities of products are required. Recently, examples of up to 12 syntheses of oligonucleotide phosphodiester (Pon et al., 1999) and phosphorothioate (Pon et al., 1998) sequences on the same reusable supports have appeared, and further improvements in this area are expected.

CHEMICAL REQUIREMENTS FOR SOLID-PHASE SUPPORTS

A wide variety of different insoluble support materials have been developed for different applications. The ideal support should contain an appropriate chemical group on its surface that can be selectively coupled, usually through a linker arm, to the first monomer unit. Normally, supports for oligonucleotide synthesis are purchased with primary amino group functionality, although hydroxyl and carboxyl derivatized supports may also be obtained. The amount of surface derivatization (loading) on the support determines the maximum amount of product that can be prepared. Supports with loadings of 100 to 1000 μmol/g or more are available for the synthesis of either peptides or small molecules (Winter, 1996). Oligonucleotide synthesis, however, is almost always performed using nucleoside loadings of less than ~100 μmol/g; optimum results are obtained on supports with less than ~40 μmol/g of nucleoside, because the efficiency of coupling decreases as the number of molecules on the surface increases. Because the amount of support can usually be increased to accommodate the scale required, supports with loadings >50 μmol/g are not commonly used. The lower coupling efficiencies obtained with higher loaded supports can actually make it counterproductive to use these materials in most automated DNA synthesizers.

The structure of the compound(s) used to join the surface of the support to the first nucleoside is also of critical importance (Fig. 3.1.3). This attachment is generally composed of two distinct portions. The first portion is the spacer that connects the active functional group (usually NH_2 or OH groups) to the matrix of the insoluble support. This spacer can be as simple as a single methylene group (e.g., aminomethyl polystyrene) or it can be a lengthy alkyl or alkyloxy chain (e.g., long-chain alkylamine CPG). Generally, a long chain is preferred to distance the terminal functional group from the support's surface. Usually, the supports are sold with a satisfactory spacer, but sometimes additional spacers are added to change the terminal functional group or to increase the overall length (Katzhendler et al., 1987; van Aerschot et al., 1988; Arnold et al., 1989).

A second difunctional molecule is then required to connect the amino group on the support to the first nucleoside unit. In oligonucleotide synthesis, this structure is commonly referred to as a linker or linker arm. In peptide synthesis and combinatorial synthesis, this structure is referred to as either a handle or anchor, terms not usually associated with oligonucleotide synthesis. In oligonucleotide synthesis, the linker arms are usually dicarboxylic acids, such as succinic acid (Pon et al., 1988; Damha et al., 1990; Bhongle and Tang, 1995) or hydroquinone-*O,O*-diacetic acid (Pon and Yu, 1997a), that connect the nucleoside to the support via ester and amide bonds. The length, rigidity, and hydrophobicity of the linker arm can affect coupling efficiency (Katzhendler et al., 1989), and the chemical stability restricts the conditions that can be used during synthesis. This affects the choice of protecting groups. Most linkers for oligonucleotide synthesis are resistant to acidic conditions and cleavable by basic conditions. This allows the most popular combination of protecting groups—acid-labile 5′-dimethoxytrityl (DMTr) groups and base-labile *N*-acyl and cyanoethyl phosphate protecting groups—to be used. As will be discussed later, the speed with which the linker arm can be cleaved is also an important consideration. Strategies that require removal of oligonucleotide-protecting groups without cleavage from the support also require linker arms that are either very stable or removable using conditions orthogonal to the deprotection conditions. Finally, different linker arms can be used to prepare oligonucleotides with terminal end modifications, such as 3′-phosphate, amino, carboxyl, thiol, or other substituents.

The chemical properties of the rest of the surface should be either inert or capable of being made inert by silylation, benzoylation, or other similar passivating treatment (Pon, 1993; Tang and Tang, 1997). This is because residual groups, such as amino, hydroxyl, or silanol groups, can also react with phosphoramidite derivatives. This creates failure sequences coupled to the support through either phosphoramidate or phosphodiester linkages and lacking the correct 3′-nucleoside. When coupling reactions are monitored by quantitation of the orange dimethoxytrityl cation released during detritylation, the formation of such failure sequences can be deduced from apparent coupling yields of >100%. Fortunately, the phosphodiester linkages to the surface of the support are difficult to hydrolyze, and these failure sequences are not released from the support by the usual cleavage conditions (Pon et al., 1988).

The hydrophobicity of the support's surface is another consideration. Supports that are quite hydrophobic, such as polystyrene and benzoylated polymethacrylate, are sometimes preferred over supports such as CPG and polyethylene glycol (PEG), which have hydrophilic surfaces (McCollum and Andrus, 1991; Tang

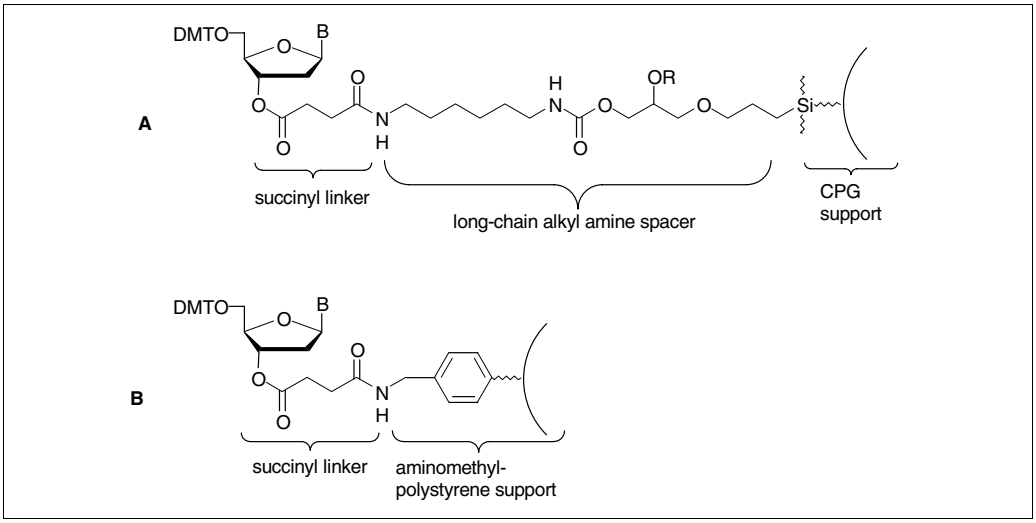

Figure 3.1.3 The structure of the two most commonly used solid-phase supports for oligonucleotide synthesis. (**A**) LCAA-CPG. (**B**) Aminomethyl polystyrene (nonswelling and highly cross-linked). In both cases, a nucleoside is attached to the amino group of the support through a succinic acid linker, which can be cleaved by ammonium hydroxide after the synthesis. DMT, 5′-dimethoxytrityl (DMTr).

and Tang, 1997). All phosphoramidite-coupling reactions are sensitive to moisture contamination, and such contamination is presumed to be more easily washed off the hydrophobic supports. This may allow greater synthesis efficiency with smaller excesses of reagent; however, quantitative comparisons are difficult to make, and both rigid polystyrene and CPG supports are widely used.

PHYSICAL AND CHEMICAL PROPERTIES OF SOLID-PHASE SUPPORTS

The accessibility of the support's surface to incoming reagents is probably the most important consideration when choosing the physical properties of the insoluble support. Although a greater surface area provides higher capacity, increased porosity must be balanced against steric restrictions and rate-limiting diffusion. The following sections discuss the major classes of solid-phase supports, categorized by the type of surface accessibility.

Liquid-Phase Supports

Liquid-phase supports are high molecular weight polymers that can be completely dissolved in the solvents required for synthesis but can be precipitated or crystallized in other solvents or solvent conditions. When they are dissolved in solution, coupling reactions on liquid-phase supports are performed in the same manner as conventional solution-phase synthesis. After completion of the coupling reaction, however, the liquid-phase support is precipitated by adding a solvent in which it is insoluble. The resulting precipitated support can then be filtered off and washed free of excess reagents in the same manner as other insoluble supports. After the washing step, the support is redissolved in the appropriate solvent, and the synthesis continued. Alternately, dialysis or ultrafiltration can also be used to remove low molecular weight impurities. The most widely used liquid-phase supports are PEG polymers (Bonora, 1995) with average molecular weights varying between 5,000 and 20,000, although cellulose acetate (Kamaike et al., 1988) and poly(*N*-acryloylmorpholine) polymers (Bonora et al., 1996) have also been used. These supports are soluble in solvents such as dichloromethane, pyridine, and acetonitrile but insoluble in solvents such as ethers and alcohols. Nucleoside loadings of ~100 to 200 μmol/g are generally obtained, and the purified yield of oligonucleotide 8- to 20-mers is about 100 mg per gram of starting support (Bonora, 1995).

The advantages of having a homogeneous solution include lower costs, because less reagent excess is required, and the ability to use spectroscopic methods (UV/VIS, NMR, Fourier transform-IR) to monitor the reactions and quality of the immobilized products. Furthermore, the method does not require any elaborate instrumentation because the reactions and precipitation/filtration steps are performed in ordinary glassware. Consequently, liquid-phase supports were some of the earliest supports to be used (Cramer et al., 1966; Hayatsu and Khorana, 1966). The method, however, is not easily automated, and each chain extension cycle requires several hours to perform. Moisture contamination must also be scrupulously avoided, because the PEG supports are very hydrophilic. Nevertheless, this method is suitable for larger scale oligonucleotide synthesis, when cost is more important than speed.

Gelatinous Polymer Supports

The first insoluble supports developed were polystyrene-divinylbenzene polymers with only a small amount (1% to 5%) of cross-linking (Pon, 1993). These supports could swell up to five times their dry volume in nonpolar solvents, such as dichloromethane, to provide a large surface area and loading capacity (0.1 to 1.5 mmol/g). Other swellable polyacrylamide-containing supports, with loadings up to 5 mmol/g, have also been developed (Winter, 1996). In these supports, up to 99% of the reactive sites are located inside the bead. They are classified as gelatinous polymer supports because of the gel-like environment in which the reactions take place (Rapp, 1996). These supports perform very poorly when used for solid-phase oligonucleotide synthesis because they don't swell satisfactorily in the polar solvents required and because reagent diffusion into and out of the supports is slow. Therefore, the swollen beads can be used only in batch reactors and not in continuous flow synthesizers (Belagaje and Brush, 1982; Ito et al., 1982; Ohsima et al., 1984). Only the very earliest oligonucleotide syntheses were attempted on these types of supports; and with one exception (Montserrat et al., 1994), their present use is restricted to peptide synthesis.

Macroporous Supports

The difficulties mentioned above were overcome by the development of rigid macroporous

supports. These supports are based on inorganic materials, such as silica gel and CPG, or highly cross-linked polymers, such as polystyrene or polymethacrylate. Well-defined pores are created in these supports to increase the surface area and loading capacity. They do not become swollen with solvent and have permanent porosity. Their rigidity allows them to be used in packed continuous-flow columns, and their properties are very similar to the packing materials used in HPLC separations. The maximum loading possible on the rigid supports, however, is much less than on the swellable supports.

Silica gel and porous glass supports are ideal nonswelling materials that are readily derivatized using techniques developed by the glass fiber and chromatography industries. CPG beads, which are stronger and easier to handle than is silica gel, are preferred and are available in three particle sizes—125 to 177 μm, 74 to 125 μm, and 37 to 74 μm—with large (75 to 4000 Å in diameter) and very uniformly sized pores. The maximum pore diameter distribution is only ±10% for 80% of the pore volume. A variety of chemically derivatized CPG supports with different functional groups is available, including magnetic CPG beads. The surface area and loading depend on the pore size; the beads with larger pores have lower loadings.

Long-chain alkylamine (LCAA) derivatized CPG supports with 500-Å pores and amino loadings of ~100 μmol/g are the most commonly used. These supports are usually derivatized with 30 to 40 μmol/g of nucleoside (Pon, 1993) and are suitable for the synthesis of oligonucleotides of up to 50 to 60 bases. The 500-Å pore size begins to restrict the coupling efficiency of longer oligonucleotides because of steric factors; however, much longer oligonucleotides (100 to 150 bases) can be prepared on 1000-Å CPG supports (Efcavitch et al., 1986). Synthesis of very long oligonucleotides also benefits from a support with a low surface loading (~5 μmol/g), because it contributes to greater coupling efficiency.

Highly cross-linked rigid polymer beads with large pores have also been developed as an alternative to CPG. These were developed primarily to overcome cost and supply problems associated with CPG supports. The greater inertness of the polymers relative to CPG, especially during alkaline deprotection conditions, was also an advantage.

The first rigid synthetic polymer, introduced by Perkin-Elmer/Applied Biosystems Division (PE/ABD), was a highly cross-linked polystyrene support with 1000-Å pores (McCollum and Andrus, 1991). These supports produce excellent quality oligonucleotides, and prepacked ABI LV40 (40 nmol) and ABI LV200 (200 nmol) columns are widely used. The nucleoside capacity of the supports, however, is lower than that of CPG, and prepacked columns >200 nmol are not available.

A second rigid polymer based on a polymethacrylate vinyl alcohol copolymer with 1000-Å pores has also been used (Reddy et al., 1994b). This copolymer is sold as a chromatographic medium by both Merck and Toso-Haas, respectively, under the trade names Fractogel and Toyopearl. It can be purchased with either hydroxyl functional groups (for size exclusion chromatography) or amino functional groups (for affinity chromatography). These supports are easy to handle, durable, and inexpensive, and their loading capacity is much higher (up to 135 μmol of nucleoside/g) than either rigid polystyrene or CPG supports.

Composite macroporous supports

Composite supports have been prepared that combine the advantages of gelatinous and rigid supports. These supports are prepared by polymerizing a low cross-linked polyacrylamide inside the pores of a rigid macroporous substrate, such as silica gel, or highly cross-linked polystyrene beads. Typical capacities are between 100 and 500 μmol/g. The soft gelatinous phase is protected by the rigid carrier, and these supports can be used in a continuous flow column system. The supports, however, are fragile, and swelling differences can create unwanted fines. Although this type of support was once used for oligonucleotide synthesis by the phosphotriester method (Gait et al., 1982), present use of commercially available composite supports is limited to other fields.

Grafted Polymeric Carriers

Another method of creating hybrid supports, which combine the advantages of gelatinous supports and rigid supports, is to covalently couple or graft long polymeric chains onto the surface of a rigid support. The surface polymers are not cross-linked and are readily solvated, whereas the rigid core remains insoluble. The low thickness of the surface layer and the absence of cross-linking increase the rate of mass exchange and allow a large number of functional sites to be introduced for nucleoside attachment (up to 160 μmol/g). These supports can, therefore, be used in continuous flow column synthesis because the supports are me-

chanically stable, do not show significant swelling, and allow reagents to be removed using short wash steps.

The first example of this type of support in oligonucleotide synthesis (by the phosphodiester method) was a polystyrene-polytetrafluoroethylene (PS-PTFE) graft copolymer. This was prepared by ^{60}Co irradiation of PTFE beads and vapor-phase styrene deposition (Potapov et al., 1979). Another coated Teflon support, in the form of fibers not beads, was also commercially available in the 1980s (Bower et al., 1987; Duncan and Cavalier, 1988). More recently, PS-PTFE beads have been found very satisfactory for oligonucleotide synthesis using the phosphoramidite method (Birch-Hirschfield et al., 1996).

Another group of widely used graft copolymers are the polyethylene glycol-polystyrene (PEG-PS) tentacle polymers produced by Rapp Polymere under the TentaGel trade name (Rapp, 1996). These supports are prepared by anionic polymerization of ethylene glycol on hydroxyl derivatized cross-linked polystyrene. Copolymers with PEG chains of about 3000 Da are considered optimal, but the polymerization process can produce PEG chains as large as 20,000 Da. Unlike the previous hydrophobic PS-PTFE copolymers, the PEG-PS copolymers have an insoluble polystyrene core and a hydrophilic PEG coating. The relative amounts of material in the coating and the core are also quite different. The PS-PTFE supports have between 2% and 10% polystyrene as the surface coating, whereas the PEG-PS supports have 70% PEG as the coating and only 30% polystyrene as the core. Therefore, the properties of the TentaGel resins are mostly dictated by the PEG coating. The supports swell considerably (3 to 5 times dry volume) in solvents that dissolve PEG, but owing to the insoluble core, they are suitable for both batch and continuous flow processing. The gel-like environment surrounding these supports allows coupling reactions to proceed in a manner similar to solution-phase reactions. This environment presumably allows cyclization reactions to proceed much more efficiently than on CPG supports, and circular oligonucleotides of up to 32 bases have been prepared (Alazzouzi et al., 1997). A number of different TentaGel resins are commercially available with different functional end groups, particle sizes (ranging from uniformly sized 10 µm beads to 750 µm macrobeads), and loading capacities (0.25 to 1.3 mmol/g) for all types of solid-phase and combinatorial synthesis (Winter, 1996). The high capacity of these supports has found particular use in large-scale (200 to 1000 µmol) oligonucleotide synthesis (Wright et al., 1993).

Nonporous Supports

Rigid nonporous beads without surface copolymerization have also been used as supports of oligonucleotide synthesis, although the capacity is two to three orders of magnitude less than similar porous supports. Nonporous silica beads allow long oligonucleotides to be prepared with high coupling yields because of the absence of restrictive pores (Seliger et al., 1989, 1995). The very small diameter (1.5 µm) particles required to provide an acceptable surface loading (2 µmol/g), however, made this support very difficult to work with. Similar handling problems also occurred when nonporous 4.5-µm magnetic Dynabeads were used (Albretsen et al., 1990).

A more practical application for nonporous supports is the synthesis of immobilized oligonucleotides. Although a large number of methods have been developed to immobilize previously synthesized oligonucleotides on insoluble supports, it is simpler to synthesize the oligonucleotide directly onto the support required for the final hybridization assay. This type of synthesis requires a stable linker that can withstand the conditions used to remove all of the protecting groups (typically 55°C NH_4OH, 16 hr) after completion of the synthesis. The deprotected oligonucleotides left attached to the support can then be used as hybridization probes. Both nonporous glass (Maskos and Southern, 1992) and polystyrene beads, with respective loadings of 50 to 70 and 150 nmol/g, have been used. In the latter case, time-resolved fluorescence detection on single Dynosphere beads was performed (Hakala et al., 1997).

Hybridization assays using two-dimensional formats, however, are much more common than assays using beads. Consequently, a great deal of effort has gone into the synthesis of oligonucleotide arrays on flat glass and polypropylene supports. The surfaces of glass slides can be derivatized using the same techniques developed for silica gel and CPG supports. Typical surface loadings of 10 to 40 pmol/cm^2 are obtained (Maskos and Southern, 1992; McGall et al., 1997), although one account of ~166 pmol/cm^2 was reported when phosphoramidite reagents were reacted directly with surface silanol groups (Cohen et al., 1997). Oligonucleotide synthesized with permanent linkages to quartz fibers has also been used as a DNA sensor (Uddin et al., 1997).

Recently, polypropylene sheets have been used as solid-phase supports for oligonucleotide synthesis (Matson et al., 1994, 1995 Wehnert et al., 1994). Polypropylene has the advantages of greater flexibility, physical strength, and chemical stability at high pH. It also has low nonspecific adsorption of biomolecules. One report, however, has mentioned an incompatibility between polypropylene supports and the tetrahydrofuran (THF) solvent commonly used in capping and oxidation reagents (Weiler and Hoheisel, 1996).

The inertness of this polymer makes chemical derivatization difficult. Amino-modified supports are prepared by exposure of the polypropylene surfaces to ammonia vapor inside a radio frequency plasma (RFP) generator (Chu et al., 1992). This results in an amino group loading of about 15 to 25 nmol/cm^2. Oligonucleotides have been attached to these amino groups, either directly via phosphoramidate linkages (Matson et al., 1994) or through intermediate spacers with terminal hydroxyl (Shchepinov et al., 1997) or amino groups (Weiler and Hoheisel, 1996) to give supports with respective loadings of 10, 0.3, and 0.03 to 0.09 nmol/cm^2. Efficient hybridization requires optimization of both loading density and spacer length, because duplex formation can be inhibited by too close spacing and by spacers that are either too short or too long.

Polypropylene can also be chemically derivatized without requiring an RFP generator. Bromination using *N*-bromosuccinimide and 2,2′-azobisisobutyronitrile followed by amination with long-chain diamines or amino alcohols has recently been described, but no surface loadings were reported (Seliger et al., 1995). Oxidation of polypropylene with chromium(VI) oxide followed by borane-tetrahydrofuran complex and H_2O_2/NaOH treatment has also been used to produce hydroxyl-derivatized polypropylene tapes. Direct reaction of phosphoramidites to this tape yielded a nucleotide loading of 7 nmol/cm^2. These polypropylene tapes have been used to prepare a 200-base-long polythymidylic acid sequence and overlapping one-dimensional arrays (Bader et al., 1997a,b).

Filter Disks, Membranes, and Sintered Blocks

This section deals with supports whose physical properties are not easily categorized. Paper filter disks probably represent the cheapest and most readily accessible insoluble support for oligonucleotide synthesis. These supports contain cellulose fibers that have many hydroxyl groups available for oligonucleotide attachment and that are resistant to all of the chemical conditions required for oligonucleotide synthesis. Paper filter disks were ideal supports for synthesizing multiple oligonucleotides simultaneously using the "segmental solid-phase" procedure, because they could be easily labeled and sorted (Frank et al., 1983; Matthes et al., 1984; Ott and Eckstein, 1984; Frank, 1993).

Another important innovation was the development of commercially available MemSyn DNA synthesis supports (Perseptive Biosystems). These contain a membrane-based support made of porous PTFE and coated with an aminopropyl linker. These membranes are sealed inside specially designed low dead volume disposable housings, which resemble common syringe filters, and have loadings of either 50 or 200 nmol. The membranes are easier to handle than are particulate supports, and mass-produced synthesis cartridges are presumably more reliable and easier to manufacture. The large pore diameter of these filter membranes (0.2 µm or 2000 Å) allow both large and small oligonucleotides to be synthesized.

Finally a new process, developed at NASA, has been used to derivatize polyethylene (Devivar et al., 1999). In this process, gaseous amine radicals are used to aminate porous polyethylene sintered blocks (FlowGenix, Webster, TX). The amino functionalized sites can then be derivatized with nucleosides for oligonucleotide synthesis. This technology allows supports in the form of plugs and disks to be produced, which should be more convenient to handle than are particulate supports.

This discussion clearly indicates that a wide range of insoluble supports have been developed for an increasing number of different applications. Selection of an appropriate support requires consideration of both the chemical properties and the physical characteristics of the support. The size and shape of support beads can vary from small uniformly sized or irregularly sized particles to large macrobeads. Supports in sheet and plate form are amenable to extremely sensitive isotopic or fluorescent detection schemes. High-density arrays or "gene chips" are also emerging as important tools for gene expression studies. Supports in membrane or foam formats provide the synthesis capacity of porous beads but greatly simplify the handling and manufacturing steps required to mass produce ready-to-use synthesis cartridges.

SUPPORT DERIVATIZATION: NUCLEOSIDE AND LINKER ARM COUPLING STRATEGIES

Nucleosides are attached to the surface of the support through an intermediate linker arm, whose design must be carefully considered. The linker arm should allow easy nucleoside attachment to the support and be compatible with all of the conditions required for synthesis. Furthermore, the linker must be designed to accommodate different cleavage and deprotection strategies. The many different types of possible linkages can be classified into four groups for strategies that require either:

1. cleavage from the support with concomitant or postcleavage deprotection;
2. deprotection of the immobilized products with optional postdeprotection cleavage;
3. deprotection of the immobilized products with no cleavage from the support; or
4. linkers that impart terminal end modifications to the oligonucleotide products.

The following section discusses how different linkers have been use in the first three strategies. The use of different linkers and insoluble supports in the synthesis of end-modified oligonucleotides will be discussed in future units.

Linker Arms Cleaved after Synthesis

Succinyl linker arm

The most commonly used linker arm in oligonucleotide synthesis is succinic acid (Fig. 3.1.3). This linker was used in the early 1970s and has remained very popular because of low cost and ease of incorporation (Yip and Tsou, 1971). Both succinyl dichloride (Sharma et al., 1992) and succinic anhydride have been used as starting materials, but the anhydride is greatly preferred because of its easier handling. A suitably protected 2′-deoxyribonucleoside can be succinylated at either the 5′- or 3′-hydroxyl position, and the resulting 5′- or 3′-*O*-hemisuccinate is coupled to an amino- or hydroxyl-derivatized support. Alternatively, the support can be succinylated first and then coupled to a nucleoside (Damha et al., 1990). This method has the advantage of not requiring the synthesis of an inventory of succinylated nucleosides. Coupling of a nucleoside to a succinylated support, however, is more difficult and usually gives lower nucleoside loadings than does attachment of a presynthesized nucleoside 3′-*O*-hemiester. The coupling reactions between the succinate and the nucleoside or support have usually been performed using carbodiimide coupling reagents, such as dicyclohexylcarbodiimide (Montserrat et al., 1993), 1-(3-dimethylaminopropyl)ethylcarbodiimide (Pon et al., 1988), and diisopropylcarbodiimide (Bhongle and Tang, 1995), and required coupling times between 1 and 24 hr. A faster coupling reaction—involving reaction of a nucleoside-3′-*O*-hemisuccinate with 2,2′-dithiobis(5-nitropyridine) (DTNP) and dimethylaminopyridine (DMAP) followed by addition of triphenylphosphine (TPP) and LCAA-CPG—can reduce the coupling time to 2 to 30 min (Kumar et al., 1996). Extremely fast coupling of a nucleoside-3′-*O*-hemisuccinate to LCAA-CPG can be obtained using a variety of phosphonium or uronium coupling agents and DMAP. These reactions are complete in the time required to add the reagent to the support ~4 sec) and allow for the possibility of automated on-line support derivatization (Pon and Yu, 1997b).

After completion of the oligonucleotide assembly, the protected products can be cleaved from the support by hydrolysis of the succinyl linker arm with either concentrated aqueous ammonium hydroxide (1 to 2 hr) or gaseous ammonia at 10 bar pressure (15 min; Boal et al., 1996). Faster hydrolysis can be performed by including stronger reagents, such as methylamine (Reddy et al., 1994a) or sodium hydroxide (Chow and Kempe, 1997), with the ammonium hydroxide. These reagents can reduce the cleavage time to 5 min and speed up the removal of base-protecting groups. There are, however, potential problems with the modification of cytosine bases through either aminoalkylation (Macmillan and Verdine, 1991) or deamination (Debear et al., 1987) with these reagents.

Although the succinic acid linker has been widely used for a long time, the succinyl linker is unnecessarily stable for oligonucleotide synthesis. The relatively harsh conditions required to hydrolyze the succinyl linker are incompatible with a number of base-sensitive minor bases, backbone modifications, and dye labels; and the time required to cleave the succinyl linker with NH_4OH is unnecessarily long (Alul et al., 1991; Avino et al., 1996; Pon and Yu, 1997a). Therefore, a number of more easily

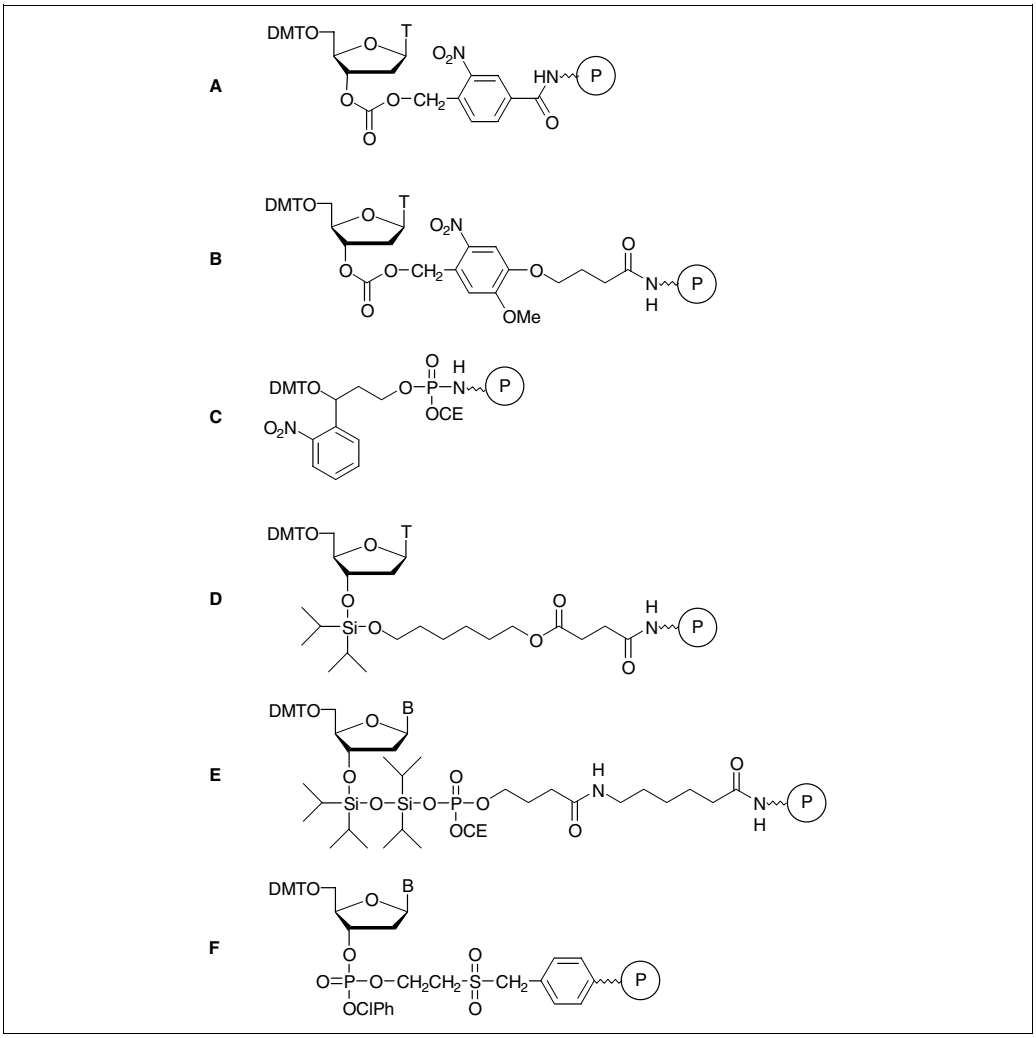

Figure 3.1.4 Structure of labile linker arms that can be cleaved under milder conditions than a succinic acid linker. (**A**) *o*-Nitrobenzyl carbonate photolabile linker arm (Greenberg and Gilmore, 1994). (**B**) 5-Methoxy-2-nitrobenzyl carbonate photolabile linker arms (Venkatesan and Greenberg, 1996). (**C**) *o*-Nitrophenyl-1,3-propanediol base photolabile linker for 3′-phosphorylated oligonucleotides (Dell'Aquila et al., 1997). (**D**) Fluoride ion labile diisopropylsilyl linker arm (Routledge et al., 1995). (**E**) Fluoride ion labile disiloxyl phosphoramidite linker arm (Kwiatkowski et al., 1996). (**F**) Benzenesulfonylethyl linker arm cleavable with triethylamine/dioxane (Efimov et al., 1983). (**G**) NPE carbonate linker arm cleavable with DBU/pyridine (Eritja et al., 1991). (**H**) 9-Fluorenylmethyl linker cleavable with DBU (Avino et al., 1996). (**I**) Phthaloyl linker arm cleavable with DBU (Avino et al., 1996; Brown et al., 1989). (**J**) Oxalyl linker, cleavable under very mild conditions (Alul et al., 1991). (**K**) Malonic acid linker for the synthesis of 3′-phosphorylated oligonucleotides (Guzaev and Lonnberg, 1997). (**L**) Diglycolic acid linker used to make 3′-TAMRA dye-labeled oligonucleotides (Mullah et al., 1998). (**M**) Hydroquinone-*O,O′*-diacetic acid (*Q*-linker), which can be used for routine oligonucleotides to improve synthesis productivity or to synthesize base-labile products (Pon and Yu, 1997a). DMT, 5′-dimethoxytrityl (DMTr).

cleavable linker arms have been investigated (Fig. 3.1.4).

Labile linker arms

Photolysis offers a very mild method for cleavage. Photolabile linker arms based on *o*-nitrobenzyl groups (Greenberg and Gilmore, 1994) have been used to synthesize oligonucleotides with 3′-hydroxyl (Fig. 3.1.4A and B), 3′-phosphate (Fig. 3.1.4C), and other 3′-end modifications. The photolysis can, however, cause small amounts (<3%) of thymine-thymine photodimers, and alkaline or other conditions still need to be employed to remove

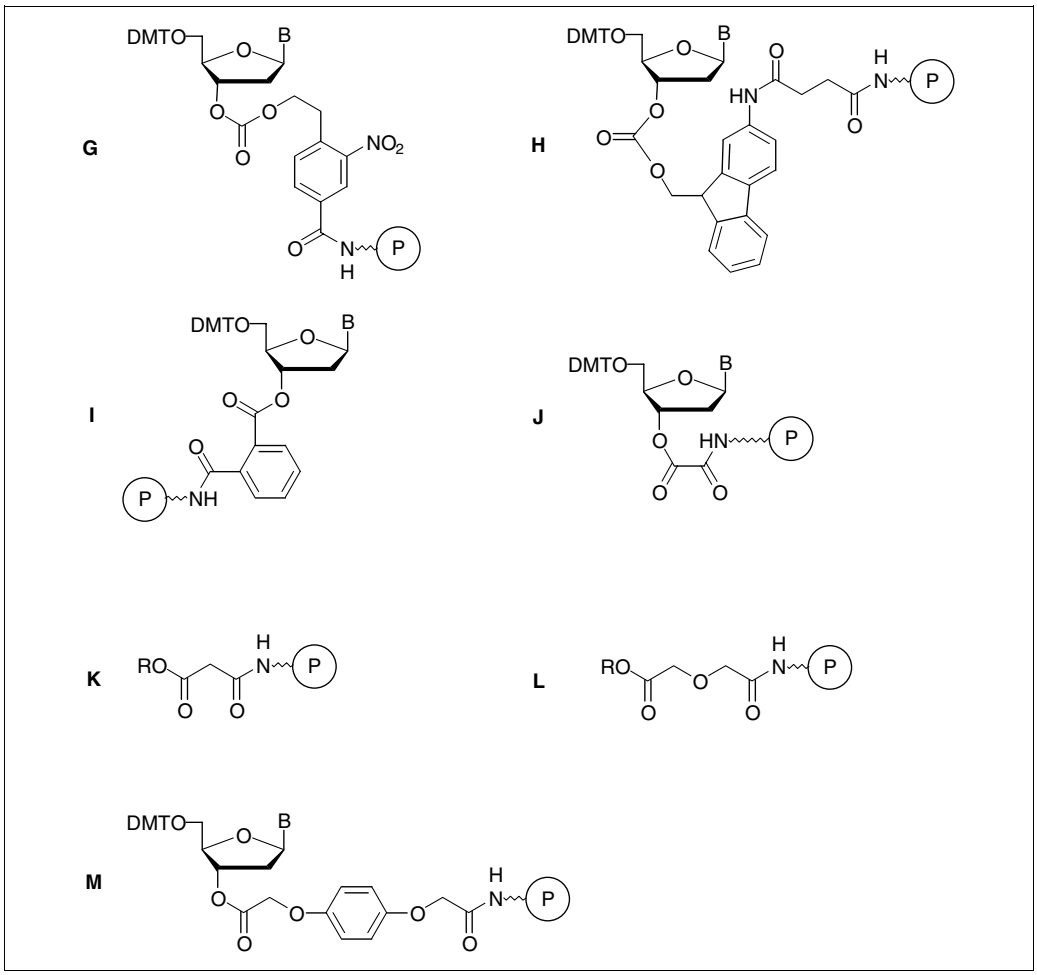

Figure 3.1.4 Continued

base-protecting groups. In addition, *N*-benzoyl-protected dA and dC nucleosides must also be avoided.

Very mild cleavage, under non-nucleophilic conditions and neutral pH, can be obtained through silyl- or disiloxyl-based linker arms (Fig. 3.1.4D and E), which are cleavable with fluoride ion. Triethylamine has been used to cleave a benzylsulfonylethanol linker arm to yield 3′-phosphorylated oligonucleotide blocks suitable for solution-phase coupling (Fig. 3.1.4F). An even more labile 2-(4-carboxyphenylsulfonyl)ethanol linker arm was considered unsuitable for phosphotriester synthesis but was not evaluated using phosphoramidite synthesis (Schwyzer et al., 1984). The non-nucleophilic base, 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU), can also be used to cleave the 2-(*o*-nitrophenyl)ethyloxycarbonyl (NPE) linker, *N*-[9-(hydroxymethyl)-2-fluorenyl]-succinamic acid (Fmoc) linker, and phthaloyl linker arms (Fig. 3.1.4G to I). When using DBU cleavage, however, thymine and guanine modification can occur if methoxy- or cyanoethyl phosphate–protecting groups are not removed before the DBU treatment. Furthermore, oligonucleotides with terminal TT sequences are not efficiently cleaved.

All of the above linker arms were either difficult to prepare or did not offer any speed advantage. In addition, the requirement for deprotection conditions or reagents different from the simple NH_4OH cleavage used in standard oligonucleotide synthesis procedures was an obstacle to the widespread adoption of any of these linker arms. More satisfactory alternatives would be other dicarboxylic acid linkers, especially if they were readily available and compatible with the derivatization and cleavage methods used for succinic acid.

The most labile dicarboxylic acid linker reported has been the oxalyl linker (Fig. 3.1.4J). This was completely cleaved by concentrated NH_4OH in only a few seconds, and cleavage with a number of other milder reagents was also possible. The oxalyl linker, however, was too

labile for routine use, and oxalyl derivatized supports had to be used within a few weeks because significant spontaneous nucleoside loss occurred (Pon and Yu, 1997a). More stable linkages have been created using either malonic acid or diglycolic acid as the linker arm (Fig. 3.1.4K and L). Treatment of diglycolic acid (Pon and Yu, 1997a) and malonic acid (Guzaev and Lonnberg, 1997) linkers with room temperature concentrated NH_4OH for 10 min was sufficient to respectively hydrolyze 68% and 90% of these linker arms, conditions that caused only 15% cleavage of the succinyl linker. The malonic acid linker arm was used in combination with a diethyl 2,2-bis(hydroxymethyl)malonate spacer to afford 3′-phosphorylated methyl phosphotriester and methylphosphonate analogues. The diglycolic acid linker has principally been used in combination with a branching spacer, such as 2-amino-1,3-propanediol, to prepare 3′-tetramethylrhodamine (TAMRA) labeled oligonucleotides, which are damaged by conventional ammonium hydroxide hydrolysis (Mullah et al., 1998). In this case *t*-butylamine/methanol/water (1:1:2) was used for cleavage from the support (20 to 60 min at room temperature) and subsequent base deprotection (1 to 3 hr at 65° to 85°C).

A more satisfactory replacement for succinic acid is hydroquinone-*O*,*O*′-diacetic acid, which is used to create a Q-linker arm (Fig. 3.1.4M). This linker is sufficiently stable so decomposition during room temperature storage is not a problem. The Q-linker, however, can be cleaved much faster than either the succinyl or diglycolic acid linkers (Pon and Yu, 1997a). For example, cleavage using NH_4OH required only 2 to 3 min and cleavage using *t*-butylamine/methanol/water was performed in only 5 min, instead of the 45 min described above. Moreover, for routine use, supports derivatized with the Q-linker can be used without any modifications to either protecting groups, reagents, or synthesis procedures (other than a reduction in cleavage time). Thus the Q-linker can serve as a general replacement for the succinyl linker in the synthesis of either unmodified or base-sensitive oligonucleotides. The main advantage of the Q-linker, however, is the improved productivity that results from the decreased cleavage time. Unlike postsynthesis deprotection, which is performed off the automated synthesizer, the cleavage step is usually performed by the instrument; and subsequent runs cannot be started until the cleavage is complete. Because typical oligonucleotide syntheses are usually complete within 2 hr, waiting an additional 1 or 2 hr for cleavage of a succinyl linker represents a significant bottleneck.

Recently, the Q-linker arm has also been included in a strategy for oligonucleotide synthesis on reusable solid-phase supports (Pon et al., 1998, 1999). In this approach, an hydroxyl derivatized support is used to form ester linkages, which can be easily cleaved and regenerated for subsequent use. The mild cleavage conditions required to release the oligonucleotide reduce damage to the support's surface and reduce the time required to prepare the support for another use. This approach will be very useful in the large-scale (>1000 kg/y) manufacturing of oligonucleotide pharmaceuticals in which the support is the most expensive single consumable.

Universal linkers

In the supports discussed above, attachment of the first nucleoside is always done separately from the actual oligonucleotide synthesis, because of the different chemistry and long coupling times required. In the past, maintaining an inventory of prederivatized supports was not problematic because of the limited number of common nucleosides. The recent development of high throughput DNA synthesizers. however, has created a need for universal supports that have the terminal nucleoside added as part of the automated synthesis. This is required not so much for inventory purposes but because manual setup of prederivatized supports is time-consuming and error prone. Universal linkers are also an advantage for the synthesis of oligonucleotides containing rare or modified bases that one wishes to incorporate at internal sites and at the 3′-end. In this way it is necessary to synthesize or purchase only the phosphoramidite derivative of the rare or modified base.

Automation of the nucleoside-coupling reaction using very fast uronium coupling reagents and DMAP is one possible approach (Pon and Yu, 1997b); however, implementation of this strategy requires construction of new DNA synthesizers with additional reagent reservoirs. A simpler approach would be to design a linker arm that could use a conventional nucleoside-3′-phosphoramidite as the first monomeric unit. It is fairly simple to design universal supports that can produce oligonucleotides with 3′-phosphate ends using either amino or hydroxyl end functions (Fig. 3.1.4A to F). Furthermore, a sulfonyldiethanol phos-

phoramidite, usually used for 5′-phosphorylation, can be used to synthesize 3′-phosphorylated sequences on any amino- or hydroxyl-derivatized support (Fig. 3.1.5G). Obtaining an oligonucleotide with a free 3′-hydroxyl terminus instead of a 3′-phosphate is essential, however, if the oligonucleotides are to be enzymatically extended (e.g., used as DNA sequencing or polymerase chain reaction primers). Removal of the terminal 3′-phosphate group introduced by the 3′-phosphoramidite reagents is usually difficult, because the negative charge(s) on phosphodiester and phosphomonoester bonds make them very resistant to hydrolysis. This difficulty can be overcome by introducing a neighboring hydroxyl group so a cyclic phosphate can form via an intramolecular attack (Fig. 3.1.6). A linker containing a single ribonucleoside in an inverted orientation, so chain extension occurs from the 2′- (or 3′-) hydroxyl position and not the 5′-position, will allow cleavage of the phosphate group via a mechanism similar to the alkaline cleavage of RNA. This will produce the desired oligonucleotide with a 3′-hydroxyl terminus and a uridine-2′, 3′-cyclic phosphate.

This strategy was first used on cellulose supports (Crea and Horn, 1980; van der Marel et al., 1982); however, because dinucleotide units with inverted 3′-2′ (3′) linkages were

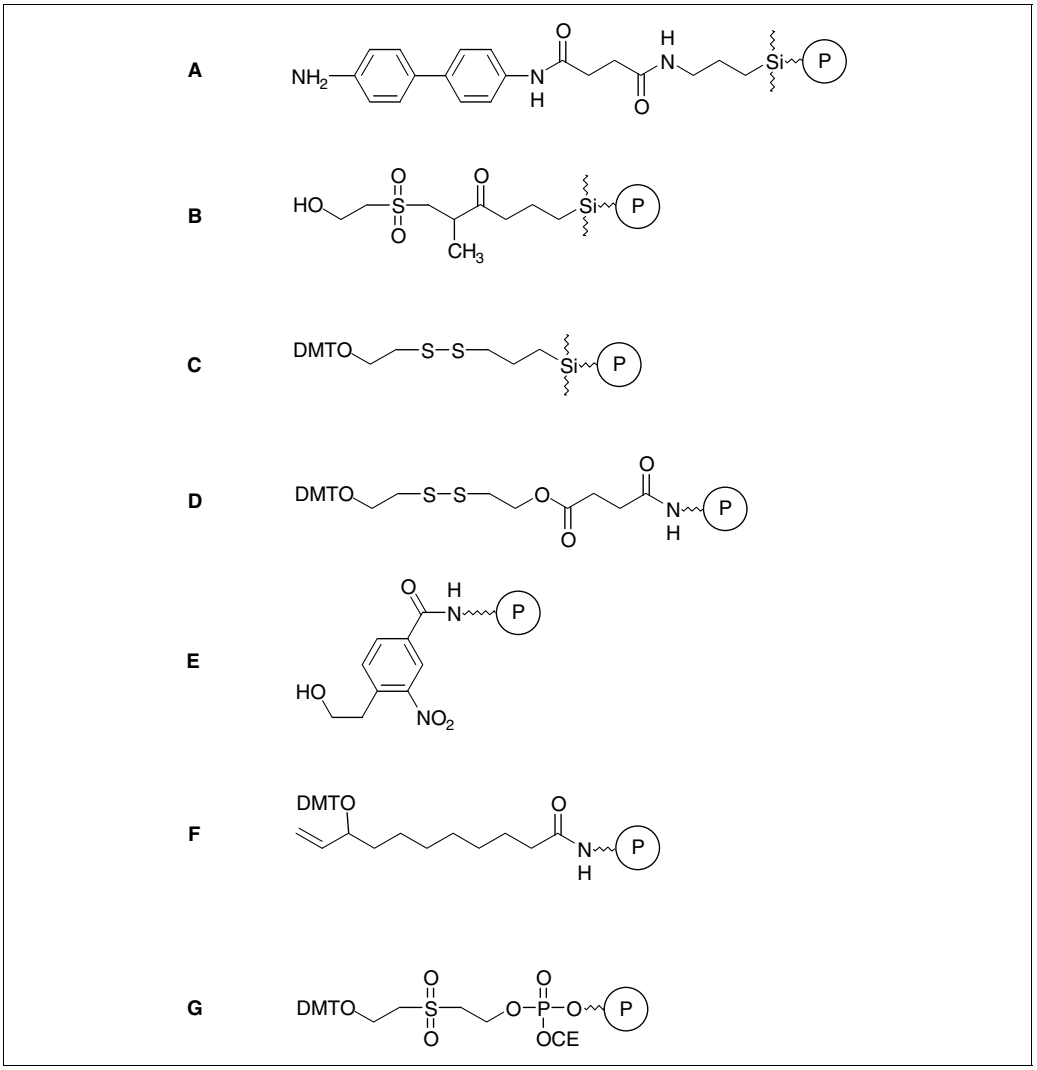

Figure 3.1.5 Structure of universal linkers for 3′-phosphorylated oligonucleotides (see Fig. 3.1.4C and K). (**A**) Benzidine linker arm (Markiewicz and Wyrzykiewicz, 1989). (**B**) Hydroxyethylsulfonyl linker arm (Markiewicz and Wyrzykiewicz, 1989). (**C**) Hydroxyethyl disulfide linker (Kumar et al., 1991). (**D**) Hydroxyethyl disulfide linker (Asseline and Thuong, 1989; Gupta et al., 1991). (**E**) NPE linker (Eritja et al., 1991). (**F**) Universal allyl linker, 9-*O*-(4,4′-dimethoxytrityl)-10-undecenoic (Zhang and Jones, 1996). (**G**) Dimethoxytrityl sulfonyldiethanol phosphoramidite linker (Bader et al., 1997b; Shchepinov et al., 1997). DMT, 5′-dimethoxytrityl (DMTr).

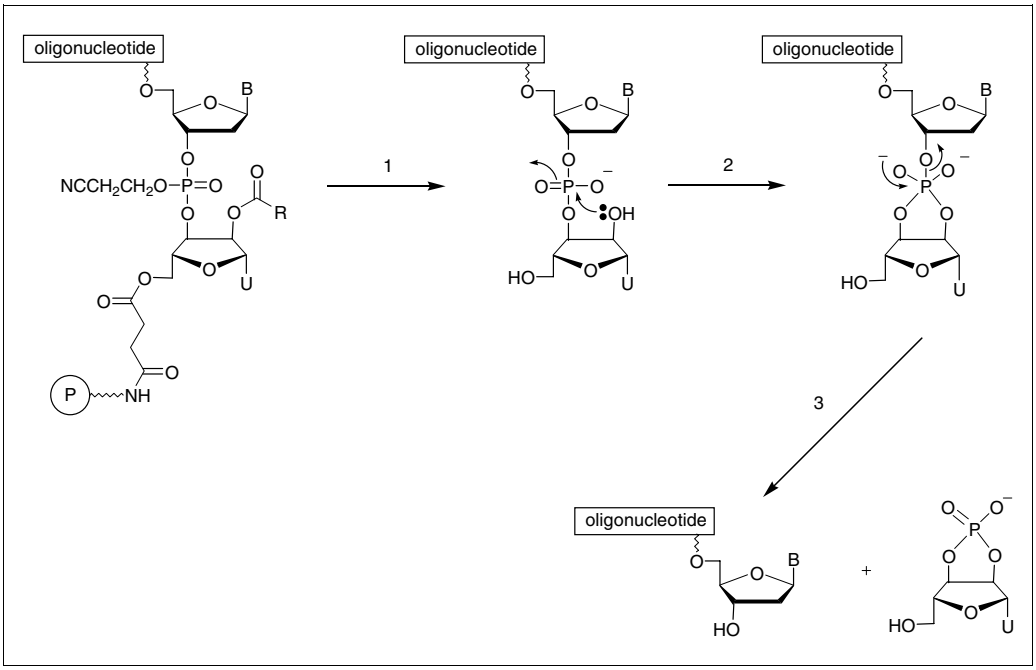

Figure 3.1.6 Mechanism of terminal phosphate cleavage in universal supports. (**1**) The first step in the cleavage/deprotection process is hydrolysis of the ester from the hydroxyl group adjacent to the first phosphate linkage. This step occurs concurrently with the removal of the cyanoethyl groups on the phosphate linkages and hydrolysis of the ester attachment to the support. (**2**) The deprotected hydroxyl group can then cyclize by attacking the phosphorus atom. (**3**) Formation of a 2′,3′-cyclic phosphodiester releases the oligonucleotide sequence with a free 3′-OH group.

prepared in solution before attachment to the support the method was not truly universal (Fig. 3.1.7A). The universal support concept was first fully examined when uridine mononucleosides were attached to CPG through 5′-succinate linkages (Fig. 3.1.7Ba to c). Oligonucleotide synthesis, using nucleoside-3′-phosphoramidites, can then be performed from the 2′ (or 3′) hydroxyl position of the uridine linker in the normal manner. Cleavage from the linker, however, involves two steps: hydrolysis of the succinyl linker to release the material from the support and elimination of the terminal uridine as the 2′,3′-cyclic phosphate. Although both steps, along with removal of base-protecting groups, can be performed simultaneously, elimination of the terminal cyclic phosphate is the rate-limiting step. After normal NH_4OH deprotection, treatment at neutral pH with lead acetate (18 hr at 37°C) can complete the terminal deblocking (Gough et al., 1983). Complete deblocking can be performed with extended NH_4OH hydrolysis, although the rate depends on the nature of the adjacent nucleoside with all ribonucleosides > dA, dG > T > dC (Debear et al., 1987). Therefore, NH_4OH deprotection conditions ranging from 16 hr at 50°C to 24 hr at 65°C were first proposed, and a subsequent paper has used 48 hr at 65°C (Schwartz et al., 1995). Attachment of a ribonucleotide through the N^4-position of a cytosine base (Fig. 3.1.7C), with subsequent chain extension from the 3′-phosphate group, has also been used (Pochet et al., 1987). In this case, cleavage was performed using 2N NaOH (10 min at 60°C); however, use of alkali hydroxides is not recommended because of possible damage to cytosine bases. A reversed uridine phosphoramidite reagent can also be used to convert previously derivatized supports into universal supports (Fig. 3.1.7D). Universal supports with *N*-benzoylcytidine linkers (Fig. 3.1.7Bd) are commercially available (Biogenex, San Ramon, CA), and the addition of 0.5 M lithium chloride to the NH_4OH reagent has been recommended for their cleavage and deprotection (15 hr at 55°C).

A ribonucleoside is not essential for a universal linker, and other diol linkers have been used. An acyclic 3-amino-1,2-propanediol linker has been reported (Lyttle et al., 1996) that uses neighboring group participation by the amino group to cleave the oligonucleotide under mild conditions (0.1 M triethylamine acetate [1 mL] plus 3% NH_4OH [40 µL], 2 hr at room temperature; Fig. 3.1.7E). This linker,

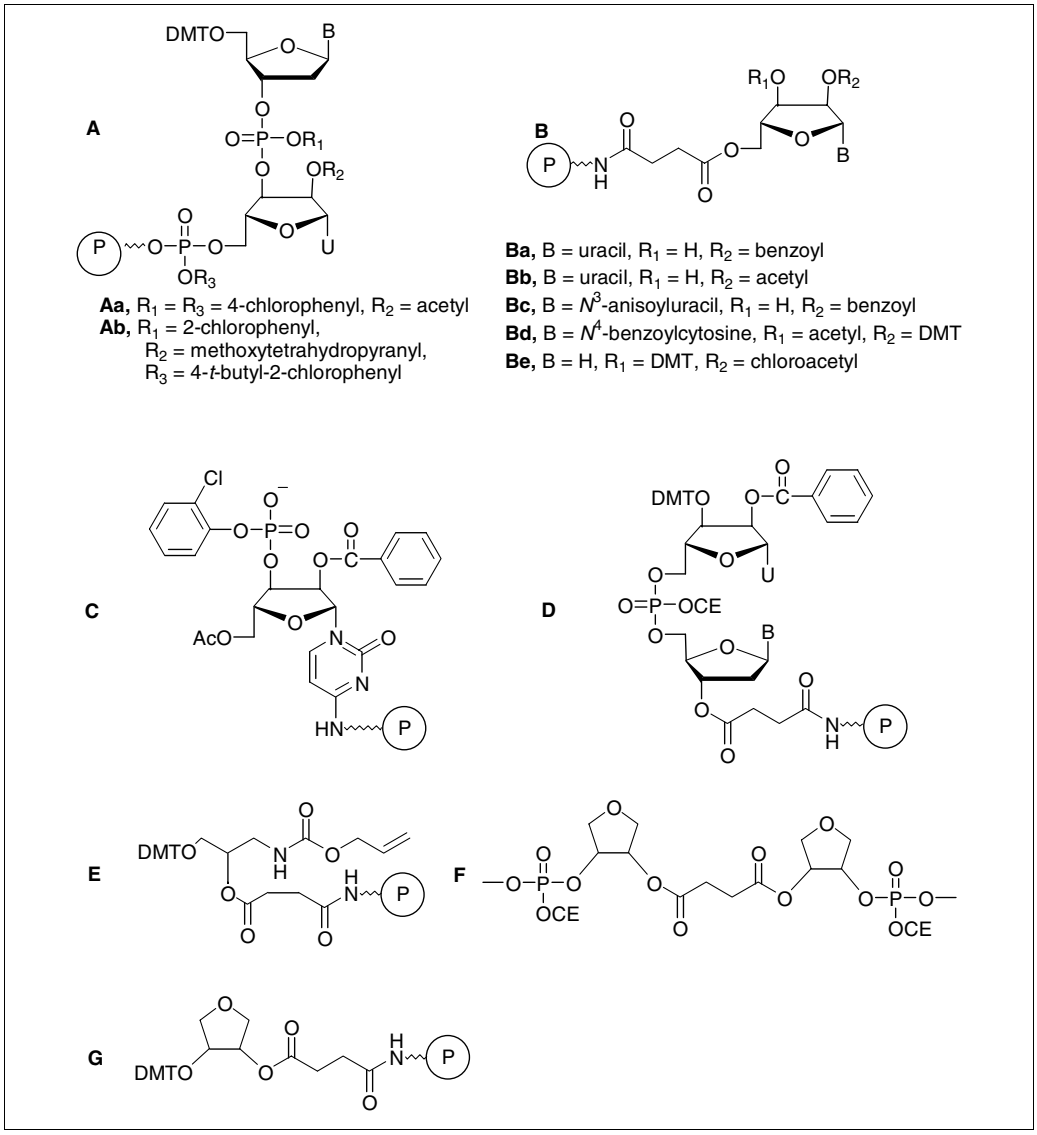

Figure 3.1.7 Universal linker arms for the synthesis of oligonucleotides with free 3′-OH ends. (**A**) First 3′-2′(3′) inverted linkages (Crea and Horn, 1980; van der Marel et al., 1982). (**B**) Universal supports based on: (**a**) 2′(3′)-*O*-benzoyluridine (Gough et al., 1983); (**b**) 2′(3′)-*O*-acetyluridine (Cosstick and Eckstein, 1985); (**c**) N^3-anisoyluridine (Debear et al., 1987); (**d**) commercially available N^4-benzoylcytidine universal support (Biogenex, San Ramon, CA); (**e**) 1,4-anhydroribitol (Scheuerlarsen et al., 1997). (**C**) Inverted linkage attached through N^4-position of cytidine (Pochet et al., 1987). (**D**) Inverted linkage obtained through use of a "universal" 5′-phosphoramidite (Schwartz et al., 1995). (**E**) Acyclic universal linker with a 3-*N*-allyloxycarbonyl-protecting group (Lyttle et al., 1996). (**F**) Special phosphoramidite linkage to allow the synthesis of a second oligonucleotide on the 5′-end of another (Hardy et al., 1998). (**G**) 1,4-Anhydroerythreitol coupled via a succinyl linker to CPG (Nelson et al., 1997). DMT, 5′-dimethoxytrityl (DMTr).

however, required a preliminary step with tetrakis(triphenylphosphine) palladium to remove the *N*-allyloxycarbonyl-protecting group, and not all of the oligonucleotide was released from the support.

The rate of cleavage has been found to depend strongly on the stereochemistry of the attacking hydroxyl group, as reported in studies that used the cyclic *cis* diol 1,4-anhydroerythritol (Scott et al., 1994; Hardy et al., 1998). These studies developed a novel linker, phosphoramidite, that allowed consecutive oligonucleotide sequences to be synthesized on the same support (Fig. 3.1.7F). Cleavage with NH_4OH (16 hr at 80°C) or $NH_4OH/MeNH_2$ (16 hr at 60°C) was recommended. The 1,4-anhy-

droerythritol linker has also been coupled to CPG supports through a succinic acid linker (Fig. 3.1.7G); these Rainbow Universal CPG supports are commercially available (Clontech, Palo Alto, CA). Deprotection and cleavage from the Rainbow Universal CPG supports was performed using either 0.5 M LiCl/NH_4OH (16 hr at 55°C) or 0.23 M triethylamine/0.5 M LiCl/NH_4OH (1 hr at 80°C) as the cleavage reagent (Nelson et al., 1997).

Deprotection of the hydroxyl group adjacent to the phosphate linkage is a requirement before this group can participate in the cyclization and phosphate group elimination reactions. Therefore, faster deprotection can be obtained when the very labile chloroacetyl-protecting group is used instead of acetyl, benzoyl, or succinyl groups (Scheuerlarsen et al., 1997). This strategy was first applied to a 1,4-anhydroribitol linker arm using a specific 2′-5′ linkage (Fig. 3.1.7Be), and cleavage of the terminal cyclic phosphate was achieved under "normal" deprotection conditions (NH_4OH for either 12 hr at 55°C or 72 hr at 22°C).

Linker Arms for the Deprotection of Immobilized Products with Optional Postdeprotection Cleavage

A number of applications require the removal of all or some of the protecting groups before the oligonucleotide is cleaved from the support (i.e., deprotection conditions orthogonal to cleavage conditions). Removal of the terminal 4′-dimethoxytrityl-protecting group is easily performed because the acidic conditions do not affect the acid-resistant linker arms most commonly used. In contrast, removal of the phosphate-protecting groups from the internucleotide linkages or the amino-protecting groups from the adenine, cytosine, and guanine bases requires special consideration if these groups are to be removed without cleaving the product from the solid-phase support. If, however, these deprotection steps are performed while the product remains immobilized (i.e., attached to the support), then the solid-phase support can provide the same handling and workup advantages as realized during the solid-phase synthesis. After these steps, the linker arm can be cleaved to release the products. In certain cases, the final product will be used while immobilized on the support, but it is still helpful to be able to cleave samples off for characterization and quality analysis.

Generally, succinyl linkers are not sufficiently stable to allow removal of any protecting groups other than *O*-methyl- or cyanoethyl phosphate–protecting groups. Succinyl linkers, however, can be left intact if different protecting group and deprotection schemes are employed. For example, hydrazine hydrate/pyridine/acetic acid can be used as the deprotection reagent if the N^6-isobutyryl-protecting group on deoxyguanosine is replaced with an N^6-(*N*′,*N*′-dibutylformamidine)-protecting group (Urdea and Horn, 1986). Base deprotection using ethanolamine can also be accomplished, but the N^4-protection on deoxycytidine must be modified to prevent N^4-hydroxyethylation (Berner et al., 1989). Allylic protection on all of the bases and on the phosphate linkages can also be removed using palladium reagents without affecting the succinyl linker (Hayakawa et al., 1990).

Attachment of a succinyl linker to a secondary *N*-methyl amino group on an intermediate linker such as *N*-methyl glycine (sarcosine), *bis*-1,6-dimethylaminohexane, or *N*-propyl polyethylene glycol (Fig. 3.1.8A to C) instead of a primary amino group (such as LCAA-CPG) creates linkages that are resistant to cleavage by the non-nucleophilic base DBU. These linkages have been used with base labile 5′-Fmoc-protecting groups to make acid-sensitive oligodeoxyribonucleotides and oligoribonucleotides (Brown et al., 1989) or with 2-(4-nitrophenyl)ethoxycarbonyl/NPE base-protecting groups to allow on-column deprotection (Stengele and Pfleiderer, 1990; Weiler and Pfleiderer, 1995). A triethylamine-resistant sarcosine-succinic acid linker arm (Fig. 3.1.8D) has also been used to prepare branched RNA and DNA/RNA chimeras (Grotli et al., 1997). In each case, after synthesis or deprotection, the products are easily released from the support by conventional hydrolysis with NH_4OH.

A stronger linkage can also be created by attaching the succinyl linker to the amino group of a base instead of a hydroxyl group (Figs. 3.1.7C and 3.1.8E and F). Alkaline hydrolysis or oxidative cleavage with $NaIO_4$ can then release the product. This strategy, however, requires two orthogonal-protecting groups for the 5′- and 3′(2′)-positions and does not offer any advantage, except for the synthesis of cyclic oligonucleotides.

A number of different linker arms have also been developed that take advantage of the resistance of phosphate ester and amide bonds to alkaline hydrolysis and allow protecting group removal before cleavage from the support. Thus thioether and thiophosphate linkers cleavable by oxidative cleavage (Fig. 3.1.8G and H), allyl

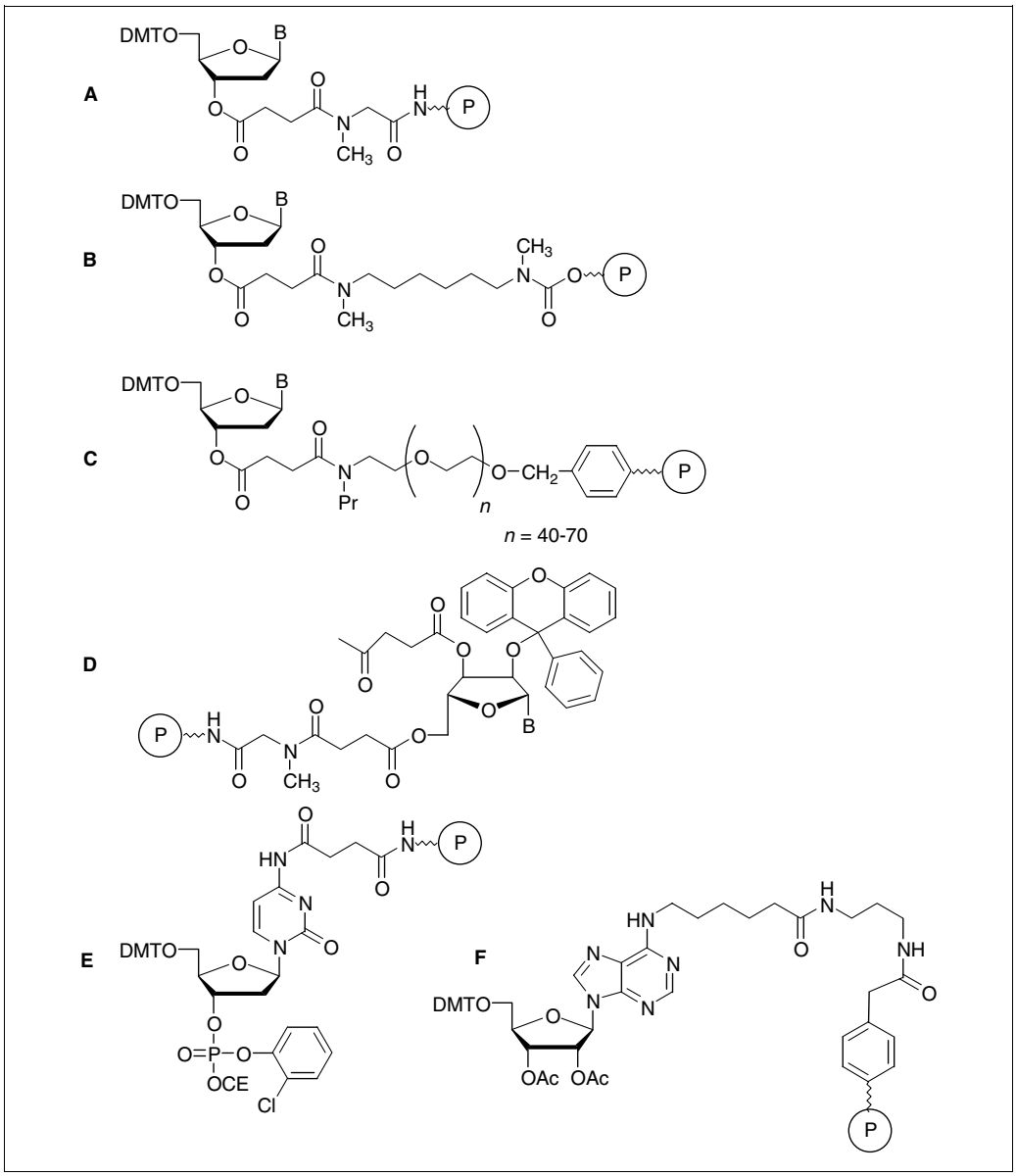

Figure 3.1.8 Linker arms that allow on-column deprotection and then optional cleavage. (**A**) Succinic acid linked to an *N*-methylglycine (sarcosine) derivatized support (Brown et al., 1989). (**B**) Succinic acid linked to 1,6-*bis* methylaminohexane spacer (Stengele and Pfleiderer, 1990). (**C**) Succinic acid linked to *N*-propyl polyethylene glycol Tentagel support (Weiler and Pfleiderer, 1995). (**D**) Succinyl-sarcosine linkage for the solid-phase synthesis of branched oligonucleotides (Grotli et al., 1997). (**E**) Linkage through the amino group of cytosine for branched and cyclic oligonucleotide synthesis (De Napoli et al., 1995). (**F**) Oxidizable solid support (Bower et al., 1987; Markiewicz et al., 1994). (**G**) Phenyl thioether linker, which is stable until oxidized into a phenylsulfone (Felder et al., 1984). (**H**) Thiophosphate linker, cleavable by iodine/water oxidation or acetic acid hydrolysis (Tanaka et al., 1989). (**I**) 3-Chloro-4-hydroxyphenyl linker for the solid-phase synthesis of cyclic oligonucleotides (Alazzouzi et al., 1997). (**J**) Linker arm produced from tolylene 2,6-diisocyanate with more stable carbamate and urethane linkages (Kumar, 1994; Sproat and Brown, 1985). DMT, 5′-dimethoxytrityl (DMTr).

linkers cleavable with tetrakis(triphenylphosphine) palladium (Fig. 3.1.5F), and phosphoroamidate linkages cleavable by acidic hydrolysis have been developed (Gryaznov and Letsinger, 1992). These supports can also be considered as universal supports because the first nucleotide is added as part of the automated synthesis. All of these methods, how-

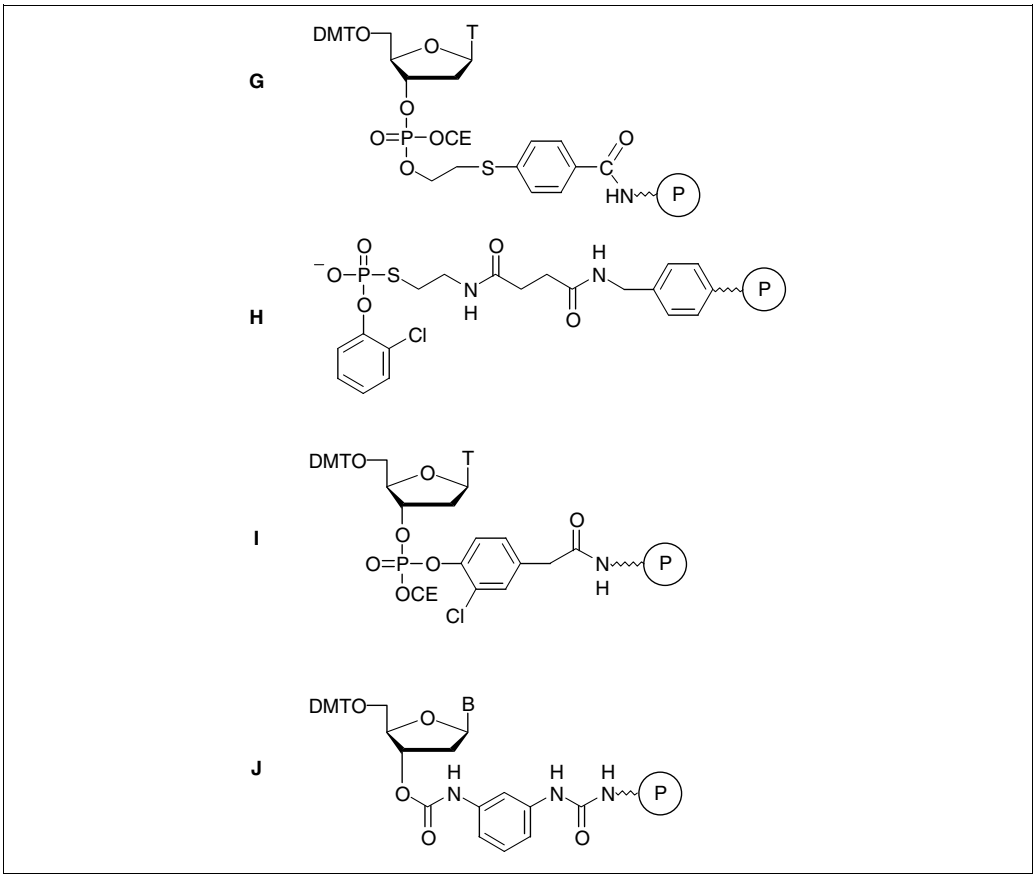

Figure 3.1.8 Continued

ever, produced oligonucleotides with a terminal 3′-phosphate instead of a 3′-hydroxyl group. An exception was the solid-phase synthesis of cyclic oligonucleotides. In this approach, 3-chloro-4-hydroxylphenylacetic acid served as both the linker arm and a phosphate-protecting group (Fig. 3.1.8I). The phosphotriester linkage was converted into a phosphodiester by selective removal of the cyanoethyl-protecting group, and then cyclization of the 5′-end to the 3′-terminal phosphodiester group was achieved using 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole as the coupling agent. The linker was then cleaved with tetramethylguanidinium *syn*-2-aldoximate (8 hr), and a circular oligonucleotide was released (Alazzouzi et al., 1997). The requirement for a 3′-phosphate has also been avoided by insertion of a disiloxyl linkage between the nucleoside and the phosphate linkage (Fig. 3.1.4E). This linkage was stable to mild base, and preliminary purification by selective cleavage of apurinic sites using triethylamine/ethanol (1:1, 3 hr at 20°C), was possible (Kwiatkowski et al., 1996). After this treatment, the products were cleaved from the support with tetrabutylammonium fluoride and then deprotected with NH_4OH.

Isocyanate reagents can react with hydroxyl groups and amino groups to produce carbamate and urethane bonds, respectively, which are more resistant to hydrolysis than are esters. Although acyclic diisocyanates have not been successful, the more rigid tolylene 2,6-diisocyanate reagent (Sproat and Brown, 1985) has been used to create a stable linker arm (Fig. 3.1.8J). The carbamate linkage can be hydrolyzed by long exposure to NH_4OH (24 to 48 hr at 55°C), whereas deprotection under milder conditions allowed the product to remain attached to the support.

Two methods have been developed for removing failure or depurinated sequences from the full-length product while both are still attached to the support. In the first method (Urdea and Horn, 1986), spleen phosphodiesterase was used to selectively degrade non-full-length oli-

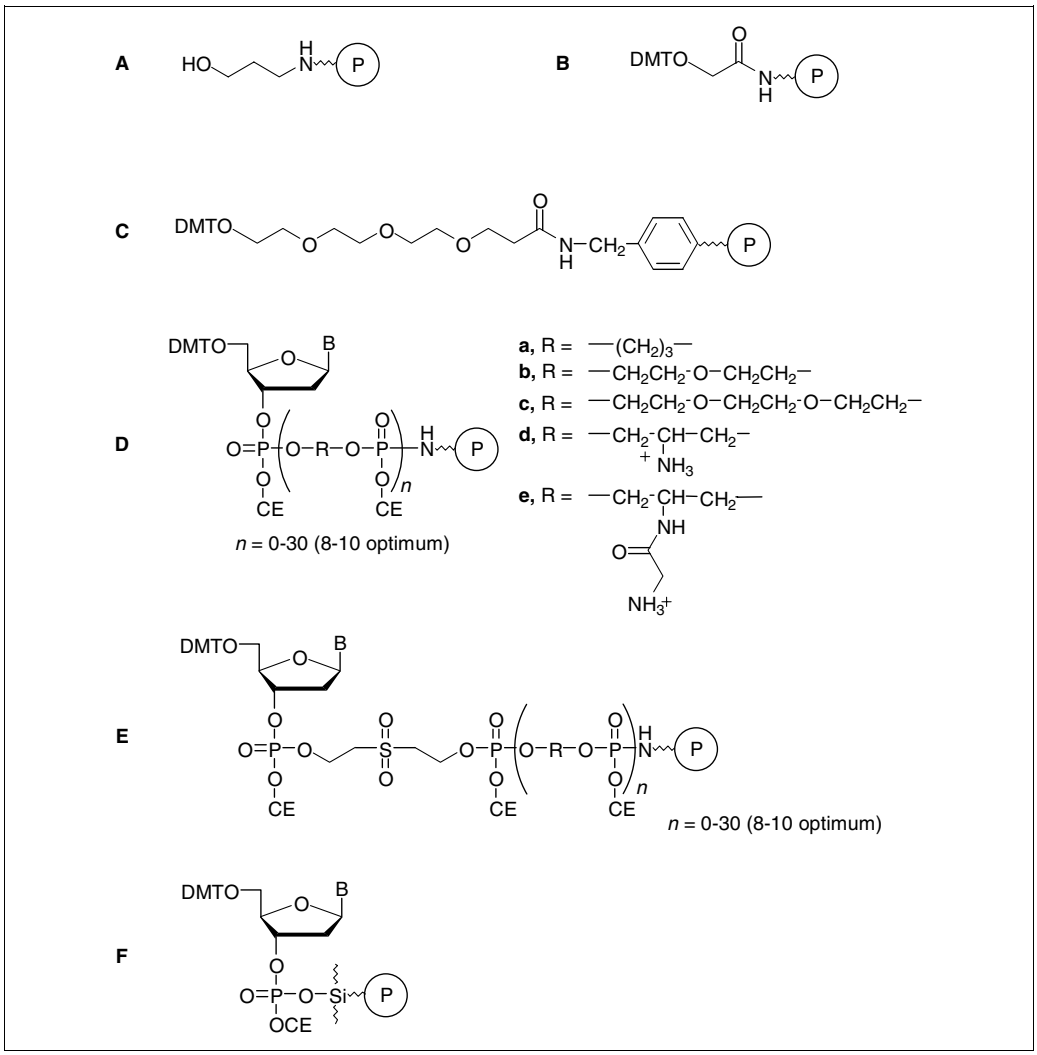

Figure 3.1.9 Linker arms for permanent attachment to solid-phase supports. (**A**) Hydroxy propylamine linker (Seliger et al., 1995). (**B**) Dimethoxytrityl glycolic acid linker (Hakala et al., 1997). (**C**) Dimethoxytrityl-4,7,10,13-tetraoxatridecanoate linker (Markiewicz et al., 1994). (**D**) Long spacer linkages prepared using repetitive coupling of various phosphoramidites (Shchepinov et al., 1997). (**E**) Cleavable spacer linkage used in conjunction with the preceding phosphoramidites to control the surface oligonucleotide density (Shchepinov et al., 1997), (**F**) Direct phosphate linkage to surface silanol groups (Cohen et al., 1997). (**G**) Diol linker formed from 3-glycidoxypropyl trimethoxysilane (Maskos and Southern, 1992). (**H**) Polyethylene glycol linkers (Maskos and Southern, 1992). (**I**) *Bis*-(2-hydroxethyl)-aminopropylsilane linker with hexaethylene glycol spacer phosphoramidites (Pease et al., 1994). (**J**) *N*-(3-(triethoxysilyl)-propyl)-4-hydroxybutyramide linker (McGall et al., 1997). (**K**) Linkage through the N^4-position of cytosine (Markiewicz et al., 1994). (**L**) Triethylene glycol ethylacrylamide linker (Markiewicz et al., 1994). DMT, 5′-dimethoxytrityl (DMTr).

gonucleotides. This method required a careful selection of protecting groups and a special capping reagent (levulinic anhydride) that could be selectively removed without exposing the full-length product (blocked by a DMTr or 5′-benzoyl group) to enzymatic digestion. In a second approach (Horn and Urdea, 1988; Kwiatkowski et al., 1996), the purification of 5′-dimethoxytritylated oligonucleotides was improved by selective fragmentation of sequences containing apurinic defects, while still attached to the support. This treatment greatly reduced the proportion of DMTr-labeled oligonucleotides that were not full-length and allowed oligonucleotides as long as 118 bases to be purified by a simple reverse-phase cartridge procedure.

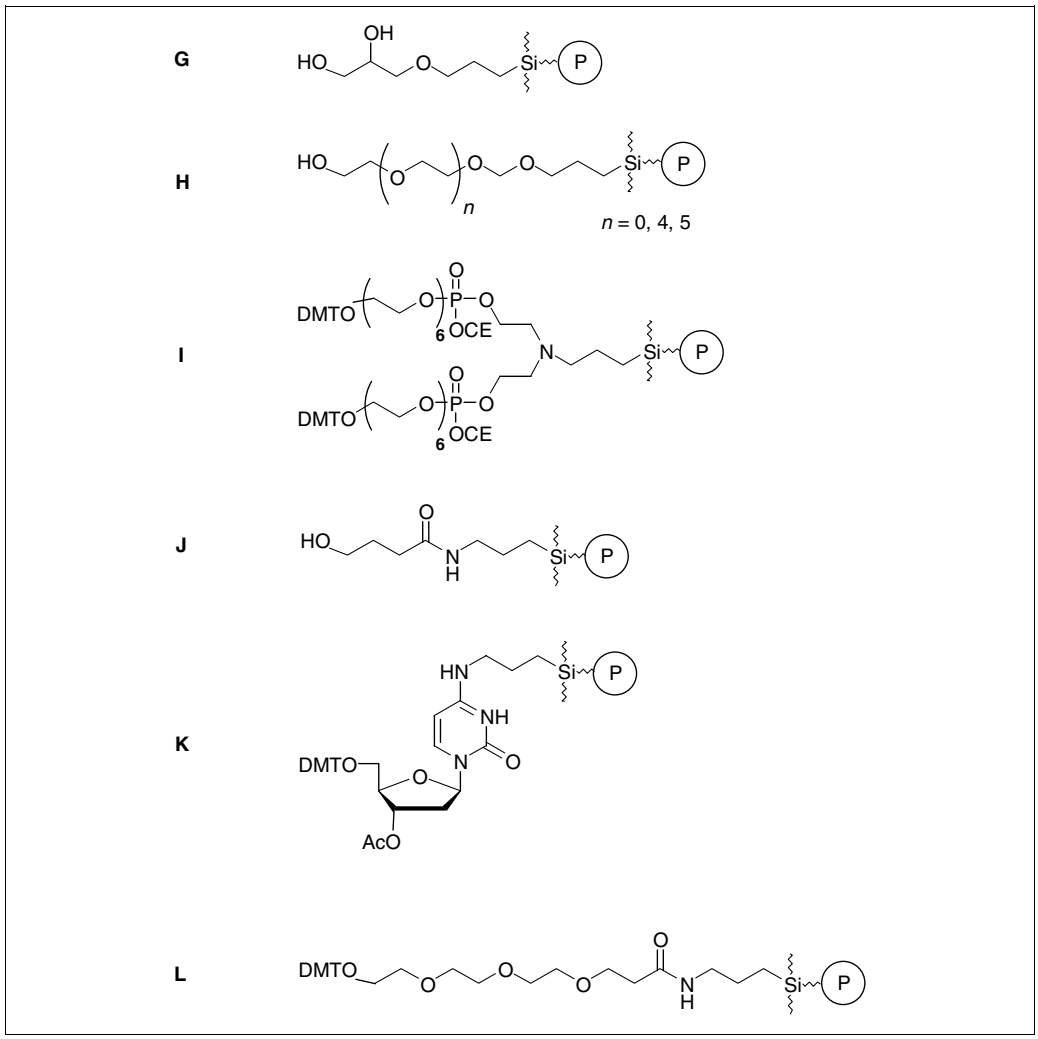

Figure 3.1.9 Continued

Linker Arms for Permanent Attachment to the Support

Linker arms for permanent attachment of the oligonucleotide to the support must be very resistant to the hydrolysis conditions used to remove protecting groups. The most common method for permanent attachment is through phosphodiester or phosphoroamidate linkages by the direct coupling of phosphoramidite reagents, respectively, to hydroxyl or amino derivatized supports. The phosphoramidate linkages are particularly common when aminated polypropylene films are used as supports (Matson et al., 1994, 1995; Wehnert et al., 1994; Shchepinov et al., 1997). Other derivatized supports have been converted into hydroxyl functionalized materials using either 3-amino-1-propanol, dimethoxytrityl glycolic acid, sodium 13-*O*-dimethoxytrityl-4,7,10,13-tetra-oxatrid ecanoate, or a variety of consecutive spacer phosphoramidites (Fig. 3.1.9A to D). These spacer phosphoramidites may also contain cleavable sulfonyldiethanol groups (Fig. 3.1.9E) or positively charged 2-amino-1,2-propandiol groups so that the surface density and charge can be modulated as well as the spacer chain length.

Although a recent publication has described the direct coupling of phosphoramidite reagents to the silanol groups on acid-washed glass slides (Fig. 3.1.9F), most glass surfaces are derivatized with alkoxysilanes to produced linker arms extending away from the surface. Several hydroxy-derivatized linkers on glass based on 3-glycidoxypropyl trimethoxysilane and ethylene glycol ethers have been described (Fig. 3.1.9G and H). Unfortunately, however, these linkers were not completely resistant to NH_4OH (5 to 10 hr at 55°C), and much of the product was lost. Other hydroxyl linker arms on glass plates have been based on *bis*-(2-hy-

droxyethyl)-aminopropylsilane or *N*-(3-(triethoxysilyl)-aminopropyl)-4-hydroxybutyramide and used in the synthesis of high-density oligonucleotide arrays (Fig. 3.1.9I and J). In these reports, base deprotection was accomplished using 1,2-diaminoethane/ethanol (1:1, 2 to 6 hr at room temperature) instead of hot NH_4OH, and no linker cleavage was reported. Linkages to CPG beads using attachments through the N^4-position of 2′-deoxycytidine or through a triethylene glycol ethylacrylamide linker have also been reported (Fig. 3.1.9K and L); however, the researchers noted that the lability of the disiloxane bond (Si-O-Si) between any silica-based support and silane linker arm is a limiting factor and, although certain resistance to aqueous NH_4OH is possible, use of pyridine/NH_4OH always results in substantial cleavage (Markiewicz et al., 1994).

CONCLUSIONS

Solid-phase oligonucleotide synthesis was once considered to be a "mature" technology limited to incremental improvements; however, new and ingenious applications for oligonucleotide based materials continue to be developed, and the role of solid-phase synthesis is clearly going to be very important in making these new materials available. Quite remarkably, the scale of these applications spans the range from the extremely minute (i.e., single molecule detection and nanoengineering) to large-scale oligonucleotide pharmaceuticals. Success in any of these areas requires a firm understanding of the chemical requirements of every step involved. It is hoped that this short review provides enough introduction to convey the power and diversity of solid-phase oligonucleotide synthesis techniques and convince the reader that new technology can be developed in even mature fields.

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