Gene Synthesis Machines: DNA Chemistry and Its Uses

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Summary. Deoxyoligonucleotides can now be synthesized rapidly and in high yield because of recent advances in nucleic acid chemistry. Key innovations include solid-phase synthesis on silica-based supports and the development of stable deoxynucleoside phosphoramidites as synthons. When incorporated into manual, semiautomatic, or automatic instruments, these new procedures can be used to prepare probes, mixed probes, deoxyoligonucleotides for priming DNA synthesis, analogues of deoxyoligonucleotides, and DNA segments containing more than 100 deoxynucleotides.

Methodologies for synthesizing DNA have improved dramatically during the past 10 years. In 1977 Amarnath and Broom concluded (*1*) that "such goals as the synthesis of a tRNA [transfer RNA] molecule or the facile preparative synthesis of genes still shimmer in the distance. . . . [T]he combined enzymatic and chemical synthesis of the gene for yeast alanine tRNA required 20 man-years of effort. The minimum time which resulted from a computer determination for the optimal path to the two complementary 75 nucleotide chains was still 11 man-years (*2*). We have a long way to go." The current state of the art is quite different. By 1981, new developments in the chemical synthesis of DNA (*3–5*) had led to procedures for synthesizing six to eight deoxyoligonucleotides simultaneously, with each segment containing at least 20 mononucleotides (*6, 7*). Moreover, compounds could be synthesized rapidly (1 to 2 days for a set), with repetitive yields being approximately 95 percent. Thus, a tRNA gene could be synthesized in a few days rather than several years. When used in conjunction with modern instrumentation technology, these procedures have led to the development of so-called gene machines for the synthesis of DNA (*8, 9*) containing more than 100 monomers (*10*). This article begins with a review of the chemistry that led to the development of gene machines. It then outlines the current and perhaps future uses for the products of this chemistry—synthetic deoxyoligonucleotides—in biochemistry, molecular biology, cell biology, and biotechnology.

The first successful synthesis of a dinucleotide containing the natural 5′–3′ internucleotide phosphate linkage was achieved by Michelson and Todd in 1955 (*11*). Over the past 30 years, three methods for synthesizing DNA have been successfully developed; these are the phosphate diester, phosphate triester, and phosphite triester methods (*12–14*). Since most synthetic DNA is now prepared by the phosphite triester method, this chemistry will be described.

DNA Chemistry

The phosphite triester method (*15*), like the phosphate triester method (*16*), originated in the laboratory of R. L. Letsinger. The initial phosphite triester procedure (Fig. 1) involved condensation of appropriately protected deoxynucleosides with dichlorophosphines, which, after oxidation to the natural pentavalent phosphate, produced the dinucleotide. This basic procedure was quite attractive because it resulted in high yields of dinucleotides relatively free of side products (compared to the diester and triester phosphate methods). However, it had several serious disadvantages. These included instability of the dichlorophosphine toward hydrolysis and toward oxidation and the need to carry out the reactions at low temperatures (−78°C). As a consequence of these limitations, syntheses had to be completed essentially in an inert gas atmosphere at −78°C while the system was accessible to a vacuum line. These conditions are neither convenient to nonchemists (the primary users of synthetic DNA) nor desirable for designing DNA synthesizing machines.

The phosphite triester approach, which solves these problems (*7*) and is now used extensively, is summarized in Fig. 2. The general synthetic strategy involves adding mononucleotides sequentially to a deoxynucleoside covalently attached to an insoluble polymer support. At the conclusion of the synthesis, the deoxyoligonucleotide is chemically freed of blocking groups, hydrolyzed from the support, and purified to homogeneity by polyacrylamide gel electrophoresis (PAGE). Two key innovations were the successful development of silica gel as an insoluble support (*3, 4*) and the discovery of deoxynucleoside phosphoramidites as efficient, stable synthons (*5, 17*).

The most efficient matrices for DNA synthesis so far developed are silica-based supports such as Vydak (*3*), Fractosil (*7*), and controlled pore glass (CPG) (*18*), which has been reported to give repetitive yields exceeding 99 percent (*10*). Although the precise reason for the superiority of these supports is unknown, these supports are rigid and do not swell (unlike organic-based polymers)—qualities that are assumed to lead to more efficient mass transfer of reagents and diffusion of solvents throughout the matrix. In addition, these supports are ideal for automation in a machine because they can be placed in a chamber, exposed to various chemical reactions without loss of material through chemical degradation of the matrix, and freed of reagents and solvents during each synthesis cycle merely by filtration. The preparation of compound 4 containing a deoxynucleoside covalently joined to silical gel through an amide bond involves reacting a deoxynucleoside containing a 3′-*p*-nitrophenylsuccinate ester with an amino group attached to the support. Usually the ratio of reagents is adjusted so that 10 to 100 μmol of deoxynucleoside are present per gram of silica. These substitution levels are convenient for synthesizing either the minimal quantities of DNA required for biological studies or the milligram amounts required for biophysical research.

Addition of a mononucleotide to compound 4 requires the following four steps (Fig. 2 and Table 1): (i) removal of the dimethoxytrityl protecting group with acid to form compound 5; (ii) condensation with the appropriately protected deoxynucleoside 3′-phosphoramidite

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(compound 8); (iii) acylation or capping of unreactive deoxynucleoside; and (iv) oxidation of the phosphite triester to the phosphate triester. Thus, the synthesis proceeds stepwise in a 3′ to 5′ direction by the addition of one nucleotide per cycle. Because the current coupling efficiencies are so very high, there is no need to use dinucleotide or trinucleotide synthons for preparing even long deoxyoligonucleotides (50 to 100 mononucleotides each). As a result, instruments for synthesizing DNA can therefore be quite simple because only four ports (A, C, G, and T monomers) are needed to generate all possible DNA sequences instead of the 16 or 64 ports that would be required if dimers and trimers were used.

The key intermediates for sequentially adding mononucleotides to the polymer support are appropriately protected deoxynucleoside 3′-phosphoramidites (compound 8). These synthons are remarkably stable toward oxidation and hydrolysis, which makes them ideal for DNA synthesis. The condensation step is completed by dissolving the appropriate deoxynucleoside 3′-phosphoramidite and tetrazole in acetonitrile and adding this solution to the substituted silica support (the synthesis is complete within a few seconds). Although several weak acids such as tetrazole and amine hydrochlorides were found to activate these amidites (presumably either by formation of a protonated amidite or the corresponding tetrazolide or chloridite), tetrazole was the acid of choice (*5*) because it is nonhygroscopic and can be easily purified by sublimation. (Other acids, including amine hydrochlorides, readily absorb water; this would quench the condensation step and lead to considerably lower DNA yields.)

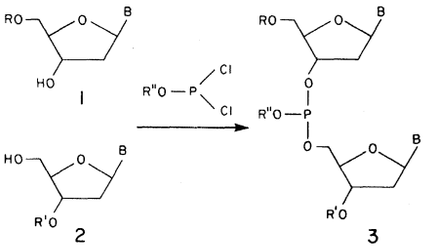

Fig. 1. The initial phosphite triester approach to DNA synthesis. Abbreviations: B, thymine or appropriately protected adenine, cytosine, or guanine; R, R′, and R″, protecting groups.

Recent research directed toward improving these synthons has focused on designing a series of color-coded trityl groups as an aid to manual synthesis (*19*), testing various dialkylamino groups for enhanced stability (*17, 20*), and examining various base-labile phosphorus-protecting groups (*21, 22*). Among these developments, the β-cyanoethyl group has been proposed as superior to the methyl group for protection of phosphorus (*21*) because it can be removed with concentrated aqueous ammonia, the same reagent currently used to remove the base-protecting groups. For some time, however, reports in the literature have shown that the methyl group can also be removed in the same manner (*8, 23*). The β-cyanoethyl group has also been found to be unstable to reagents used during the capping step, which means that high coupling yields can be maintained only by increasing the concentration of nucleoside phosphoramidite used as the synthesis progresses (*24*).

Recently, a more significant development concerning the methyl-protecting group has been reported for synthetic DNA. Gao *et al.* have shown that synthetic deoxyoligonucleotides in the triester form (after oxidation of the methoxyphosphite) are agents potentially capable of methylating the nitrogen group on thymidine residues (*25*). The percentage of alkylation is small and should be of little concern to those who use synthetic DNA as probes, as primers for DNA synthesis, or for incorporation into plasmids during cloning experiments. However, the consequence of methylation will be of importance to researchers who use synthetic DNA for biophysical studies. Nevertheless, there are two important points worth considering. First, because the condensation times used in this article were considerably longer (20 minutes) than those recommended (5 minutes) for this chemistry (Table 1), the extent of alkylation is likely to be correspondingly larger. Second, as shown by Gao *et al.*, rigorously pure DNA can be obtained by using reversed-phase high-performance liquid chromatography (HPLC) to separate the contaminating alkylated DNA from the major product (unalkylated DNA). These points demonstrate that additional research is needed to identify phosphate-protecting groups free of the limitations currently observed with either methyl or β-cyanoethyl groups.

Perhaps the most significant recent improvement in the procedure outlined in Fig. 2 has been the development of new exocyclic amino-protecting groups that stabilize deoxyadenosine toward the acid-catalyzed depurination that occurs during detritylation (Table 1, step 1). The consequence of depurination is a lower overall yield. Of the protecting groups examined to date, the most promising appear to be the succinoyl (*26*) and various amidines (*27, 28*), in that an approximately 20-fold stabilization has been achieved in each case. All the evidence now available suggests that the introduction of these protecting groups on deoxyadenosine will improve the overall, isolated yields of final products free of depurinated DNA and will extend synthetic capabilities to even longer DNA's.

Once a synthesis has been completed, the deoxyoligonucleotide is freed of protecting groups and isolated by PAGE or reversed-phase HPLC (*4, 7*). Silica containing the reaction product is first treated with triethylammonium thiophenoxide in dioxane to remove the methyl groups from internucleotide phospho-

Table 1. Chemical steps for one synthesis cycle. Multiple washes with the same solvent involve filtration between wash steps. Each step volume is 1 ml except where otherwise indicated. Total time per cycle for manual synthesis is usually about 15 to 17 minutes as described. Through optimization of various parameters, however, most machines have a cycle time of 7 to 10 minutes using the same reagents. In step 5, for each μmole of deoxynucleoside attached covalently to silica gel, 0.4*M* tetrazole (0.2 ml) and 0.1*M* deoxynucleoside phosphoramidite (0.2 ml) are premixed in acetonitrile. I_2 solution is THF:lutidine:H_2O (2:2:1, by volume) containing 0.2*M* iodine. Abbreviations: DMAP, dimethylaminopyridine; THF, tetrahydrofuran.

Step	Reagent or solvent	Purpose	Time (min)
1	Dichloroacetic acid in CH_2CL_2 (2:100, by volume)	Detritylation	3
2	CH_2Cl_2	Wash	0.5
3	Acetonitrile	Wash	1.5
4	Dry acetonitrile	Wash	1.5
5	Activated nucleotide in acetonitrile	Add one nucleotide	5
6	Acetonitrile	Wash	0.5
7	DMAP:THF:lutidine (6:90:10, weight/volume/volume) 0.1 ml acetic anhydride	Cap	2
8	THF:lutidine:H_2O (2:2:1, by volume)	Wash	1
9	I_2 solution	Oxidation	1
10	Acetonitrile	Wash	0.5
11	CH_2Cl_2	Wash	0.5

triesters. This step is followed by treatment with concentrated ammonium hydroxide to hydrolyze the ester linking the deoxyoligonucleotide to the support and to remove protecting groups from the exocyclic amino groups. Alternatively, concentrated ammonium hydroxide can be used as a one-step procedure for removing the methyl (*8, 23*) or other phosphorus-protecting groups (*21*), the exocyclic amino protecting groups, and the deoxyoligonucleotide from the polymer support. The final deprotection step is removal of the 5′-dimethoxytrityl group with 80 percent acetic acid.

In many cases, a successful synthesis may not be achieved simply because protecting groups are not properly removed. This can happen if ammonium hydroxide is improperly stored for some time at room temperature in a loosely sealed bottle. Under these conditions, much of the free ammonia will escape. Concentrated ammonium hydroxide should be stored tightly sealed in a freezer and opened only briefly during transfers to DNA samples. The reaction mixture containing deprotected deoxyoligonucleotides is then fractionated by PAGE. With a slab gel, as many as eight compounds can be purified simultaneously. The appropriate deoxyoligonucleotides are visualized with ultraviolet light (Fig. 3) and eluted from the gel by standard procedures. When synthetic DNA is being used for probing gene libraries, directed mutagenesis studies, or cloning experiments, gel purification is adequate. For various biochemical or biophysical studies on synthetic DNA (*29*), though, a more vigorous purification by reversed-phase HPLC is recommended (Fig. 4). This procedure removes DNA that is incompletely deprotected, depurinated, or alkylated. Any of these contaminants may interfere with various biochemical experiments with synthetic DNA and should be removed.

An innovation in the phosphite triester method is the in situ approach (*30, 31*). The procedure is most useful for synthesizing DNA manually, although it also has been adapted to fully automatic machines. This approach (Fig. 5) involves synthesis in situ of appropriately protected deoxynucleoside phosphoramidites. These are formed by reacting a protected deoxynucleoside with bis-diisopropylaminomethoxyphosphine and using diisopropylammonium tetrazolide as the catalyst. Bis-diisopropylaminomethoxyphosphine is very stable toward air oxidation and hydrolysis with water. Unlike dichloro or chlorodiisopropylaminophosphines (previously used to prepare deoxynucleoside phosphoramidites), it

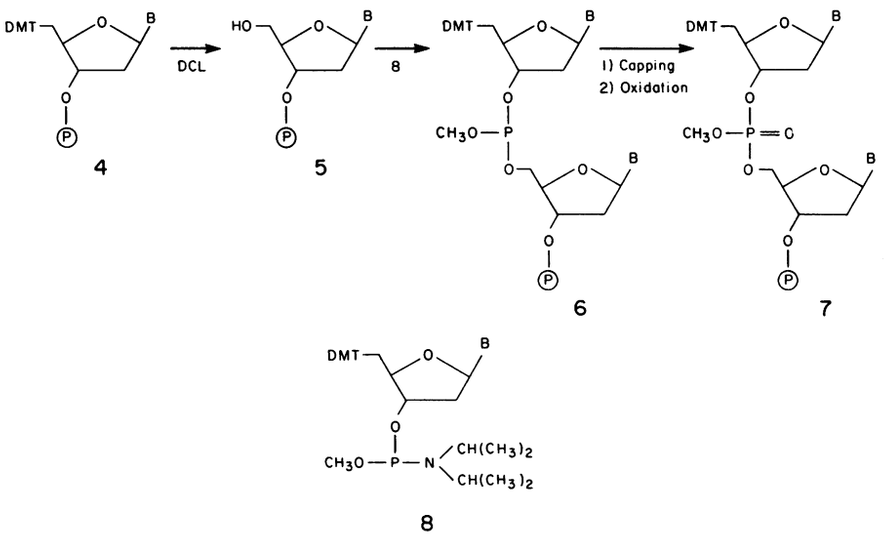

Fig. 2. Steps in the synthesis of a dinucleotide. The capping and oxidation steps are outlined in Table 1. Abbreviations: B, thymine or appropriately protected adenine, cytosine, or guanine; DMT, dimethoxytrityl; Ⓟ, silica gel or CPG support; DCL, dichloroacetic acid.

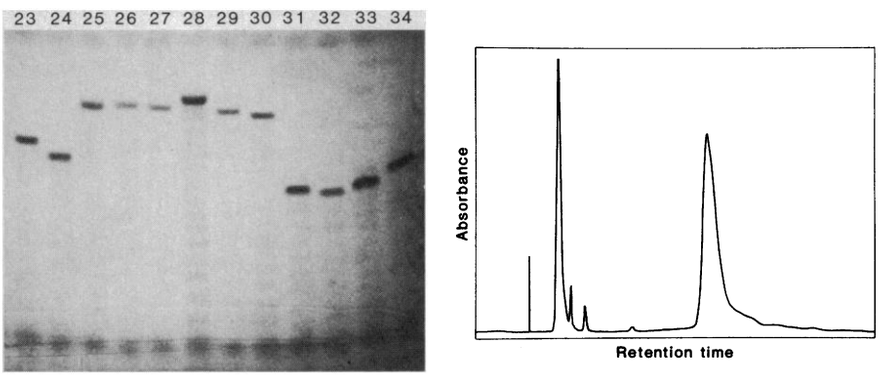

Fig. 3 (left). Purification of synthetic DNA by polyacrylamide gel electrophoresis (*59*). Deoxyoligonucleotides were synthesized simultaneously and manually on silica gel (Fractosil 500) in medium-pore, sintered glass funnels. The DNA segments (25 to 30 nucleotides in length) were separated from side products on a denaturing polyacrylamide gel by electrophoresis. The major ultraviolet absorbing band in each lane corresponds to product. The band found in all lanes near the bottom of the gel is bromophenol blue dye. The numbers identifying each lane correspond to segment numbers for a completely synthetic interleukin-2 gene. Fig. 4 (right). Purification of synthetic DNA by reverse-phase HPLC (*60*). The unpurified reaction mixture from the synthesis of d(DMT-CCTTCTAACAAGAAAACCTAGG) was fractionated on a C_{18} reverse-phase column. The eluant was a mixture of 24 percent acetonitrile and 76 percent triethylammonium bicarbonate (0.1*M*) (volume/volume). The major peak retained on the column was the product.

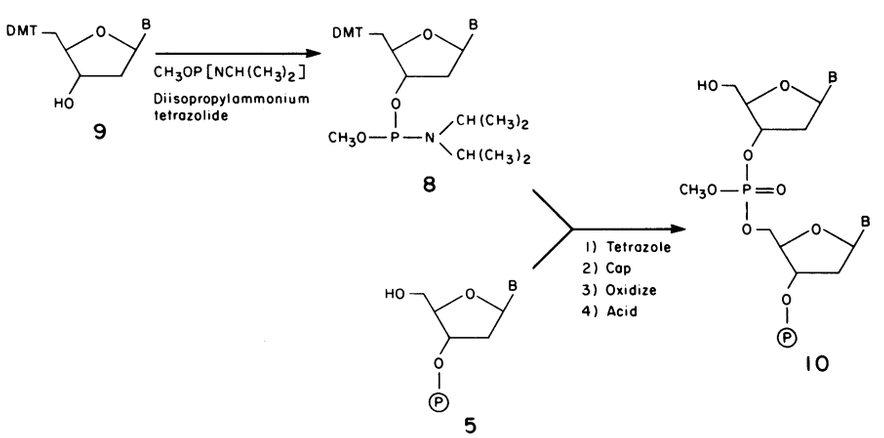

Fig. 5. In situ DNA synthesis. The cap, oxidize, and acid steps are outlined in Table 1. Abbreviations: B, thymine or appropriately protected adenine, cytosine, or guanine; Ⓟ, silica gel or CPG support; DMT, dimethoxytrityl.

can be stored indefinitely without special precautions. The deoxynucleoside phosphoramidite prepared in situ is then mixed with a deoxynucleoside covalently joined to silica, and condensation proceeds by addition of tetrazole. Other steps in the cycle are identical to those listed in Table 1. Thus, all four deoxynucleoside phosphoramidites are prepared in situ and are then added to the substituted support in the appropriate sequence to form the deoxyoligonucleotide of interest. DNA synthesis methodology has therefore evolved into a chemistry in which stable, nonhygroscopic reagents (protected deoxynucleosides, bis-diisopropylaminomethoxyphosphine, and deoxynucleoside attached to silica) can be used even by the nonchemists who lack specialized training in this area.

Gene Machines

Several types of devices are used in the synthesis of DNA. Perhaps the simplest are test tubes and sintered glass funnels (*7, 29*). If test tubes are used as reaction vessels, one begins each synthesis by placing the solid support in the test tube (compound 4). Reagents and solvents are then added sequentially and removed after a low-speed centrifugation by decantation of the liquid phase. Sintered glass funnels that contain compound 4 are attached to a water aspirator. Reagents and solvents are added sequentially and removed by filtration. When test tubes or sintered glass funnels are used to synthesize DNA, several (6 to 20) deoxyoligonucleotides can be synthesized simultaneously. Another extremely simple device used in DNA synthesis is the syringe (*32*). Silica or CPG bearing a covalently attached deoxynucleoside is placed in a syringe equipped with a filter at the base. One carries out the synthesis by successively drawing solutions into the syringe and then expelling them.

An intermediate level of technical sophistication in DNA synthesis has been achieved through the use of semiautomatic machines. Numerous designs have recently been proposed that are generally based on earlier descriptions (*3, 4*). The solid support is placed in a column joined through a series of valves and tubing to a pump and an injector loop. Usually reagents and solvents are attached to the machine, and activated deoxynucleotide synthons are added through the injector loop. The machine shuts down after one cycle, and the operator then recycles the program. A variation on this theme has been developed by Frank *et al.* for simultaneously synthesizing several deoxyligonucleotides linked to a cellulose disk support (*33*).

Completely automatic DNA synthesizers have also been described (*8, 9*). Automatic gene machines that are available commercially can usually be programmed to synthesize mixed probes containing more than one deoxymononucleotide at a predetermined position or deoxyoligonucleotides having defined sequences. In many cases these machines have also been designed to deliver unprotected samples to the operator.

Biochemical Applications of Synthetic DNA

DNA sequencing. Synthetic deoxyoligonucleotides were initially used extensively for sequencing DNA cloned in filamentous-phage cloning vectors (*34*) through use of the dideoxy sequencing method (*35*). A short synthetic DNA, complementary to part of a cloning vector such as M13, was used to prime second-strand synthesis. Because of technical limits associated with various stages of the method, one can generally obtain the continuous sequence for only 200 to 300 nucleotides of any DNA segment. Thus, the usual method for sequencing kilobase-sized DNA segments includes subcloning on the basis of the restriction map, sequencing these smaller segments, and then ordering the fragments.

A recent innovation is the stepwise sequencing of a cloned, kilobase-sized DNA segment located in the M13 vector system (*36*). Initially, a portion of the cloned fragment is sequenced. Then a new segment (10 to 15 nucleotides in length) near the 3′ terminus of the sequenced region is synthesized and used to prime a second round of sequencing. This cycle can be repeated until the total cloned fragment has been sequenced. Thus, large DNA fragments can be sequenced using multiple primers without the time-consuming process of subcloning and ordering small fragments. This procedure will probably be used in future instruments for sequencing DNA (*9, 37*).

Probes. Undoubtedly, one of the most popular applications for synthetic DNA is to probe genomic libraries for unique DNA sequences (usually genes). This material has been reviewed by Smith (*36*). Usually, short deoxyoligonucleotides (11 to 17 monomers per segment), corresponding to all possible gene sequences for a given peptide fragment, are synthesized, labeled with [^{32}P]phosphate, and used under stringent hybridization conditions to locate unique genes (*38*). In addition, an alternative approach has been developed in which a segment containing 30 to 50 mononucleotides is synthesized. If a eukaryotic gene is to be isolated, the sequence for this segment, which codes for a peptide, is based on eukaryotic codon usage, the relative stability of G-T versus A-C mismatches, and the infrequency of the dinucleotide dCpG in eukaryotic genes. Given the current ease of synthesizing deoxyoligonucleotides 30 to 50 monomers in length, the alternative approach can be used to effectively probe even genomic libraries (*39, 40*).

Another very recent development is the use of synthetic probes containing deoxyinosine at ambiguous codon positions (*41*). The analogue has been proposed as an inert base that neither destabilizes nor contributes at mismatched sites toward formation of a DNA duplex. This approach appears very promising and could prove quite useful for designing long DNA probes for regions of genes having major codon redundancies.

Directed mutagenesis. Quite often during research on genes and gene control regions, there is a need to change the sequence of bases at predetermined sites. This process can most conveniently be completed by directed mutagenesis (*42*). Although productive research in this area is in the early stages of development, a few examples illustrate progress to date. Through directed mutagenesis with synthetic deoxyoligonucleotides, it has been possible to engineer changes in protein structure, that range from a deletion of about 30 amino acids in proinsulin (*43*) to single amino acid changes in tyrosyl tRNA synthetase (*44*), dihydrofolate reductase (*45*), and interleukin-2 (*46*). An interleukin-2 mutant with a serine substituted for a cysteine led to a fully active protein that was easier to purify (presumably because the number of cysteines was reduced from three to two). Total gene synthesis affords an alternative methodology for introducing defined mutations. By selecting appropriate amino acid codons, a protein gene can be synthesized so that different DNA regions are easily accessible by restriction nucleases. In this way the amino acid sequence at any defined region of the protein can be rapidly changed or moved from one vector to another. By the use of this approach, an α-interferon having several amino acids different from any one natural subtype was constructed. When compared to any known α-interferon, it was found to have a

higher specific activity and a different, favorable spectrum of biological properties (*47*). Initial successes in these areas are quite encouraging and suggest that research on engineering proteins based on the use of recombinant DNA methodologies will grow at an ever-increasing rate for some time to come.

Now that some of the DNA-sequence elements involved in controlling gene expression have been identified, questions can be asked regarding how these gene control regions function. Directed mutagenesis utilizing synthetic DNA is one method currently being used to address this issue. Recent results on prokaryotic promoters (*48*), operators (*49*), and activators (*50, 51*) are especially interesting because the data reveal certain insights into the DNA sequence elements involved in controlling gene expression. Recent work with deoxynucleoside analogues has shown that the thymine 5-methyl group is a critical recognition site for *lac* repressor (*49*), *Escherichia coli* RNA polymerase (*52*), and cI repressor (*52*). Other research focusing on the *E. coli* ribosome binding site outlines a targeted random mutagenesis procedure that should prove to be generally useful for studying control region sequences (*53*). In this case, deoxyoligonucleotides were synthesized where the nine deoxynucleotides 5′ to the initiation codon were randomized to contain all possible base pairs. After cloning, various derived ribosome binding sites were tested for their ability to initiate protein synthesis.

Other applications. Synthetic deoxyoligonucleotides are also being investigated (*54*) for use as nonradioactive hybridization probes (*55*). The approach involves the chemical labeling of DNA probes with biotin at the 5′ terminus, hybridization of these biotinylated deoxyoligonucleotides with target DNA, and detection of the complex with avidin visualized by enzymatic methods. These procedures have been used to detect 2-fmol targets. This approach, or analogous methods, may prove useful for detecting single copy genes carrying genetic defects or infectious agents present in clinical samples.

Because deoxyoligonucleotides prepared from trivalent phosphites are oxidized to the naturally occurring pentavalent compound, a pathway is available for synthesizing various phosphorus analogues useful for biochemical research. Compounds having chirality at phosphorus owing to the presence of different stable isotopes of oxygen (*56*) or sulfur (*57*) have already proven useful for studying certain phosphorolytic enzymes. Other research has shown that alkyl phosphonates (which can also be generated from trivalent phosphites) are useful for studying biochemical processes (*58*).

Concluding Remarks

Because of recent advances in DNA chemistry, synthetic deoxyoligonucleotides can now be synthesized rapidly with the aid of manual, semiautomatic, or automatic instruments. The combination of accessibility of synthetic DNA segments plus other recent advances in molecular cloning and sequencing has made it possible to explore the molecular biology and biochemistry of the cell. For the first time, molecular biologists have total control over the sequence organization of any DNA segment being investigated. This flexibility will undoubtedly lead to major research and technological advances in the years to come.

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