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Synthetic DNA Fragments as Useful Tools in Genetic and Protein Engineering

V.A. EFIMOV, O.G. CHAKHMAKHCHEVA AND YU.A. OVCHINNIKOV

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow, USSR

Introduction

The first successful attempts to create an internucleotide bond by chemical methods were made about 30 years ago. Since that time, the synthesis of nucleic acids has constantly developed. Throughout the 1960s a few organic chemists, foremost among them Khorana's group, have steadily progressed in oligonucleotide synthesis. In the early 1970s the appearance of recombinant DNA techniques gave the research a new impetus; the 1970s and the beginning of the 1980s were marked by the rapid development of chemical DNA synthesis, which attracted more and more investigators all over the world. As a result, the modern methodology of chemical-enzymatic synthesis of polynucleotides advanced at a great rate, until it now provides an effective means of production of biologically active DNA fragments, which are universal tools for solving various problems in molecular biology, and, especially, in the area of recombinant DNAs. Recently, synthetic oligo- and poly-nucleotides have mostly been used for the construction and isolation of genes and regulatory regions, for the determination of DNA sequences and for site-directed mutagenesis.

This review outlines the application of artificial DNA fragments to genetic and protein engineering, embracing the results of Soviet researchers. As DNA synthesis is one of the main steps in such investigations, it seems reasonable to start the review with a characterization of modern DNA construction techniques, particularly with the methodology of oligonucleotide chemical synthesis.

Chemical synthesis of oligodeoxyribonucleotides

The key step in the chemical synthesis of oligonucleotides is the specific formation of an internucleotide phosphate linkage. At present there are

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three well-established approaches to oligonucleotide synthesis: these are the phosphodiester, phosphotriester and phosphite triester methods (*Figure 1*). They all allow the production of oligomers, composed of 8–20 nucleotides, widely used in many molecular biology studies, but the latter two methods can provide even longer single-stranded DNA fragments.

PHOSPHODIESTER METHOD

The diester approach, mainly developed by the Khorana team (Agarwal *et al.*, 1972), was the first to yield 10–15-mers (*Figure 2*). Suitably protected nucleoside and nucleotide components are introduced into the internucleotide condensation reaction. The first has a free hydroxyl group; the second, a free phosphomonoester group, whereas other functions are blocked. An internucleotide bond is formed with the help of a condensing reagent: initially this was dicyclohexylcarbodiimide (Gilham and Khorana,

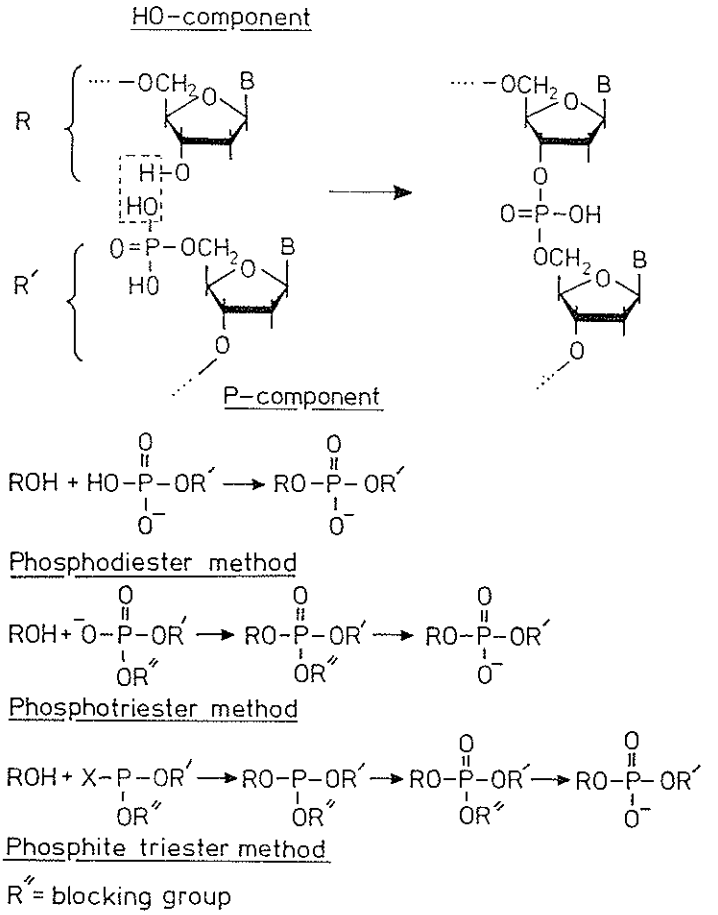


Figure 1. Three methods for the chemical synthesis of oligonucleotides.

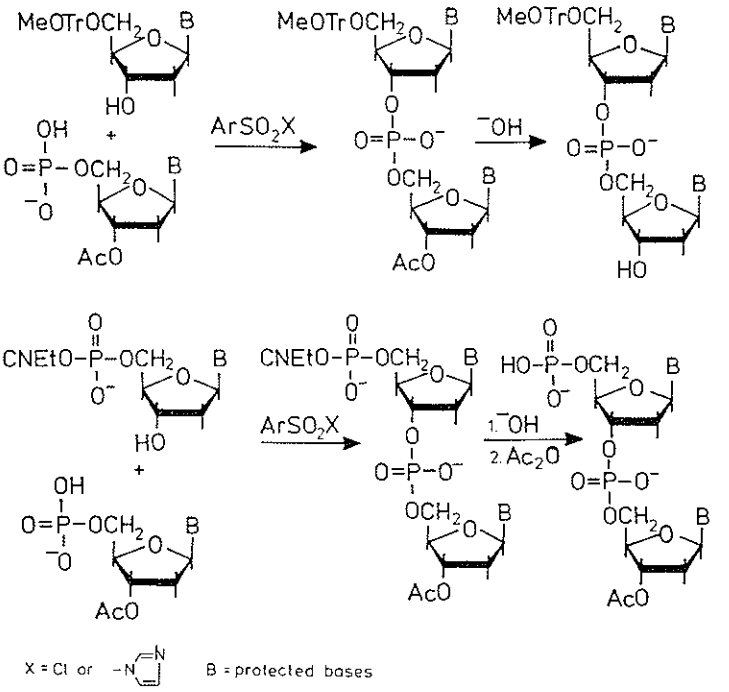


Figure 2. Chemical synthesis of dinucleotide blocks by the phosphodiester method.

1958) and then the more effective arylsulphonylchlorides were employed (Lohrmann and Khorana, 1966). Early in the 1970s, in addition to these condensing reagents, the arylsulphonylazolides—in particular, arylsulphonylimidazolide (Berlin *et al.*, 1973) and arylsulphonyltriazolide (Berlin *et al.*, 1975)—were introduced. Oligodeoxyribonucleotides synthesized by the diester method led to the synthesis of a number of functionally active DNA fragments, including two genes for yeast tRNAs (Agarwal *et al.*, 1970; Khorana *et al.*, 1976), the *lac* operator (Goeddel, Yansura and Caruthers, 1977), promoter regions (Khorana, 1979; Ovchinnikov, Efimov and Chakhmakhcheva, 1979a,b) and also small peptide genes (Efimov and Chakhmakhcheva, 1979). However, the phosphodiester approach has some disadvantages such as low yields at the steps involving internucleotide bond formation and the time-consuming procedures for purification of intermediates.

PHOSPHOTRIESTER METHOD

At the end of the 1970s an improved phosphotriester method replaced the time-consuming phosphodiester synthesis; it is characterized by the presence of an extra protecting group on the phosphate group of the nucleotide component and the internucleotide phosphates of the products (*Figures 1*

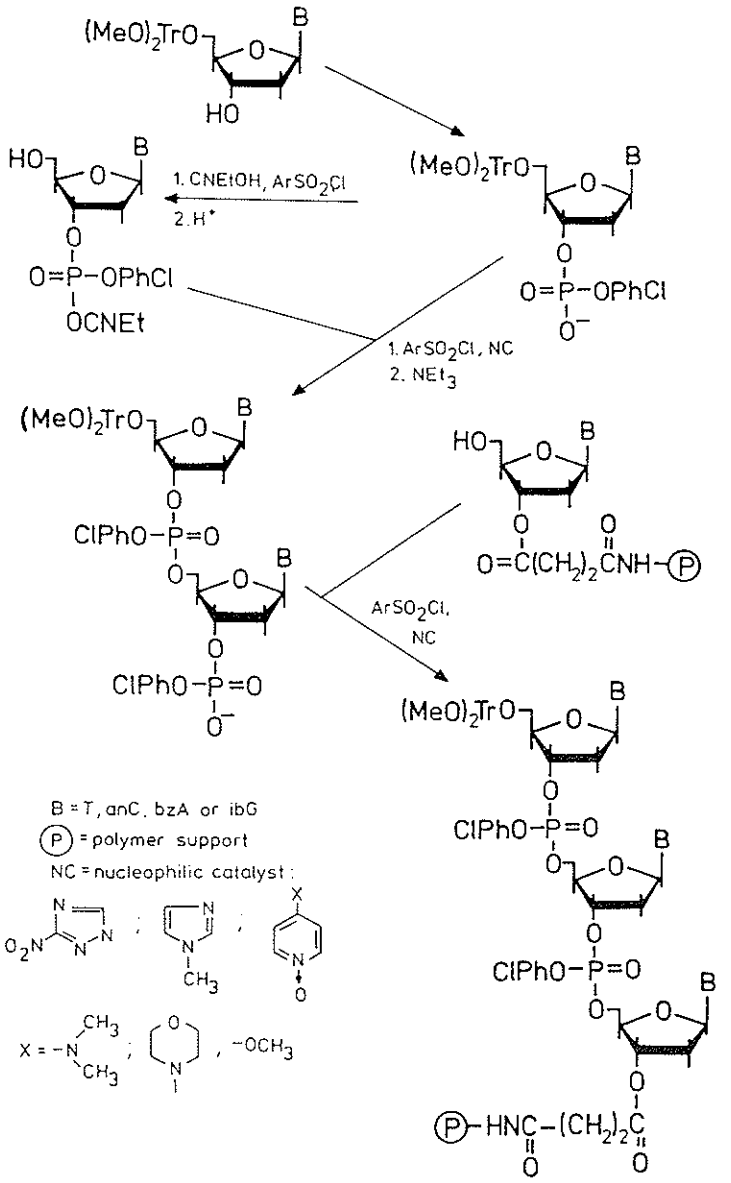


Figure 3. Synthesis of oligonucleotides by the phosphotriester method.

and *3*). Thus the component introduced into internucleotide condensation is a nucleoside phosphodiester. The presence of this phosphate-blocking group reduces the number of possible side reactions during the synthesis and increases the yield and solubility of the intermediates in organic solvents. Moreover, intermediates can be purified by fast adsorption chromatography.

Although the triester method was devised earlier than the phosphodiester

method, it was not developed extensively because of the lack of powerful condensing reagents. The introduction of arylsulphonylazolides improved the oligonucleotide synthesis technique. These condensing reagents, which had no significant advantages over arylsulphonylchlorides in the diester method, shortened the time of internucleotide condensation in the triester method and provided sufficiently high yields of oligonucleotides. Arylsulphonyl-1,2,4-triazolides (Katagiri, Itakura and Narang, 1974), -tetrazolides (Stawinski *et al.*, 1977) and -nitro-1,2,4-triazolides (Chattopadhyaya and Reese, 1980) were successfully used for oligonucleotide synthesis in many laboratories. Further improvements in the triester method were the development of block nucleotide synthesis in solution (Crea, Hirose and Itakura, 1979), use of reversed-phase high performance liquid chromatography for purification of intermediates and isolation of final products (Hsiung *et al.*, 1979; Fritz *et al.*, 1978) as well as elaboration of solid-phase oligonucleotide synthesis and its automation (Gait *et al.*, 1982). At the same time, protective groups for the various active centres of the nucleoside and nucleotide components of the reaction (Jones, 1984) and methods for deblocking of oligonucleotides (Reese, Titmas and Yau, 1978) were also improved and new approaches to the synthesis of a phosphotriester bond were developed. Thus in the early 1980s such nucleophilic catalysts as 4-dimethylaminopyridine and 1-methylimidazole were introduced into the practice of oligonucleotide synthesis. At first they were used for synthesis of short oligonucleotides with the aid of bifunctional phosphorylating reagents, namely arylphosphoditriazolides and -dichlorophosphates, to promote phosphotriester bond formation (Broka *et al.*, 1980; Knorre and Zarytova, 1980). Subsequently, Efimov, Reverdatto and Chakhmakhcheva (1982a,b) demonstrated that the addition of *N*-methylimidazole to the reaction mixture containing the 3′-phosphodiester component, 5′-hydroxyl component and arylsulphonylchloride considerably accelerates the coupling reaction. Later, mesitylensulphonyl-3-nitro-1,2,4-triazolide, plus the same nucleophilic catalyst, was also proposed as a suitable condensing agent for rapid phosphotriester synthesis (Sproat and Bannwarth, 1983). Application of 1-methylimidazole made it possible to carry out internucleotide condensations not only in pyridine, a traditional solvent for oligonucleotide synthesis, but also in various organic solvents; this simplified and unified the phosphotriester methodology and increased its efficiency (Efimov, Reverdatto and Chakhmakhcheva, 1982c).

Some recent improvements in the phosphotriester approach have increased the rate of oligonucleotide synthesis. One such improvement involves the use of oxygen-nucleophilic catalysts—4-substituted derivatives of pyridine *N*-oxide—in conjunction with the most commonly used condensing agents for the internucleotide bond formation (Efimov, Chakhmakhcheva and Ovchinnikov, 1985). These catalysts are particularly promising in solid-phase phosphotriester synthesis.

Other improvements include the enhancement of the rate of internucleotide bond formation as a result of the participation of neighbouring groups. Thus, Froehler and Matteucci (1985) have reported the use of 1-methyl-2-(2-hydroxyphenyl)imidazole as a catalytic phosphate-protecting

group; they have shown that a phenyl-protecting group bearing a 1-methylimidazole moiety in the *para* position significantly enhances the rate of phosphotriester bond formation, possibly due to the formation of an active cyclic intermediate. A similar effect was observed when 1-oxido-4-alkoxy-2-picolyl and 4-alkoxy-2-picolyl groups were used for internucleotide phosphate protection. The application of these blocking groups, which are also intramolecular catalysts, allowed a considerable increase in the rate of phosphotriester internucleotide condensation and decreased the extent of side reactions, such as sulphonation of the nucleoside 5′-hydroxyl group and the heteroaromatic lactam system (Efimov *et al.*, 1986).

Simultaneously, techniques of phosphotriester synthesis through formation of the internucleotide bond due to activated nucleotide derivatives obtained without condensing reagents, e.g. the hydroxybenzotriazole method (Marugg *et al.*, 1984), were developed.

The phosphotriester method may be carried out either in a homogeneous solution or on a solid phase. The former is preferable for obtaining oligomers in relatively large amounts (more than 0·1 μmole), whereas the latter is better in other cases. Compared with the solid-phase technique, synthesis in solution facilitates purification of intermediates and results in a higher purity of the final product, i.e. its isolation after removal of the protective groups is simplified. However, the solid-phase technique is faster and less laborious than synthesis in solution and can be automated and operated at the micro-level. At present, the phosphotriester and phosphite triester methods are widely used for synthesis on polymer supports.

PHOSPHITE METHOD

The phosphite method for oligonucleotide synthesis was initiated by Letsinger *et al.* (1975) and then adapted for solid-phase synthesis by Caruthers and co-workers (Matteucci and Caruthers, 1981). In contrast to the phosphotriester method, it utilizes activated nucleotide 3′-phosphite as a nucleotide component (*Figure 1*). At first deoxynucleoside 3′-phosphomonochloridites or monotetrazolides were employed as active derivatives. However, these substances proved to be rather impracticable for everyday work because of their instability. The problem was largely resolved when a new class of more stable deoxyribonucleoside phosphites — the deoxyribonucleotide-3′-*O*-(*N*,*N*-dimethylamino)phosphoramidites — was introduced (Barone, Tang and Caruthers, 1984). These substances become highly active reagents after activation by treatment with a weak acid, e.g. tetrazole. After coupling, the intermediate dinucleoside phosphite bond is converted into a more stable 3′-5′-phosphodiester linkage by mild oxidation (*Figure 4*).

Recent advances in the chemistry of oligonucleotide synthesis on polymer supports have enabled us to obtain 10- to 20-mers in amounts from 0·05 to 0·1 μmol in a few hours by the phosphite or phosphotriester methods. Nowadays even longer DNA fragments, up to 50- to 100-mers, can be synthesized by chemical methods (Adams *et al.*, 1983; Efimov *et al.*, 1983b; Warner *et al.*, 1984).

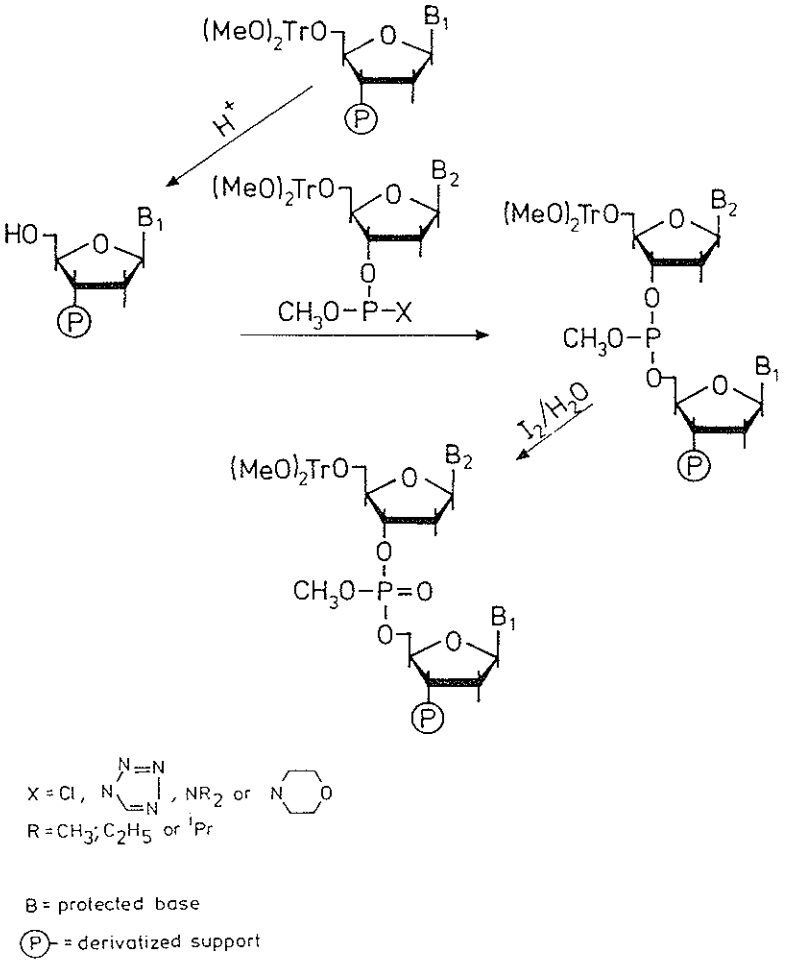


Figure 4. Solid-phase oligonucleotide synthesis by the phosphite triester method.

Methods for obtaining double-stranded DNA fragments

Modern methods of nucleic acid synthesis have paved the way for the construction of genes coding for proteins which contain over 100 amino acid residues. The first step here is chemical synthesis of oligodeoxyribonucleotides as discussed above. Synthetic oligomers are subsequently converted by enzymatic methods into double-stranded DNA fragments. More than 15 years ago Khorana (reviewed in Khorana, 1979) was the first to develop a methodology for assembling synthetic oligonucleotides into extended duplexes; this remained practically unchanged for some considerable time.

The strategy proposed by Khorana is based on the ability of oligo- and polynucleotides to form regulated double-helical complexes with single-

stranded complementary DNAs. It includes multiple linkages of oligomers with complementary overlapping base sequences by T4 DNA ligase (polydeoxyribonucleotide synthase (ATP); EC 6.5.1.1) into small duplexes with protruding single-stranded ends. The duplexes thus obtained are then assembled to give a target gene cloned in a vector molecule (*Figure 5*(A)). By this technique a number of genes, e.g. several human interferon genes of about 500 base pairs (bp), have been prepared (Edge *et al.*, 1981). Although the approach can be used for constructing long DNAs and is theoretically suitable for building genes of any length, purification of reaction products and their isolation in sufficient amounts becomes quite a problem after a number of successive ligations. Furthermore, large amounts of synthetic oligonucleotides are required, and chemical synthesis accounts for the bulk of the work. All this has encouraged the development of new procedures for DNA synthesis, to minimize the amount of chemical synthesis and to reduce the cost of starting materials.

Thus, to make synthetic double-stranded DNAs with base sequences corresponding to DNA fragments of single-stranded phages, the approaches proposed for synthesis of the gene X promoter region of bacteriophage fd (Ovchinnikov, Efimov and Chakhmakhcheva, 1979) can be employed. The natural single-stranded DNA of bacteriophage fd is readily available in large amounts. Both methods envisage the synthesis of only one strand of the target DNA fragment, using the corresponding region of the native SS phage DNA as a template. The first approach involves hybridization of several synthetic segments, which together are complementary to the whole of the promoter region, to the single-stranded fd phage DNA, followed by covalent linkage in the presence of DNA ligase, with subsequent isolation of the resultant duplex by means of S_1 nuclease (EC 3.1.30.1) (*Figure 6*(A)). The second approach, the so-called limited copying method, involves the controlled elongation of a synthetic oligonucleotide 'primer' on a single-stranded template, mediated by T4 DNA polymerase (EC 2.7.7.7) in the presence of an oligonucleotide 'stopper' for terminating the newly formed chain at the required length (*Figure 6*(B)). There are two possibilities: (1) elongation of both primers (*Figure 6*(B)I), in which case completion of the first oligonucleotide stops before the 5′-end of the 'stopper' (the latter does not compose an isolated duplex) or (2) use of an oligonucleotide 'stopper' with a blocked 3′-end (*Figure 6*(B)II), which is not a substrate for DNA polymerase (the 'stopper' comprises a synthesized duplex). The double-stranded fragments are separated from single-stranded DNA by treatment with S_1 nuclease and then cloned. It is noteworthy that these approaches allow promoter modification, in particular the insertion of photosensitive analogues of nucleotides, which have been used for structure–function investigations of *Escherichia coli* RNA polymerase (EC 2.7.7.6) (Chakhmakhcheva, Efimov and Ovchinnikov, 1980; Ovchinnikov *et al.*, 1980).

Novel synthetic techniques have widened the possibilities for chemical synthesis of 30 to 60-mers or even longer oligonucleotides, which have paved the way for new methods of DNA construction, on the one hand, and simplified the Khorana methodology, on the other hand. Recently, a pro-

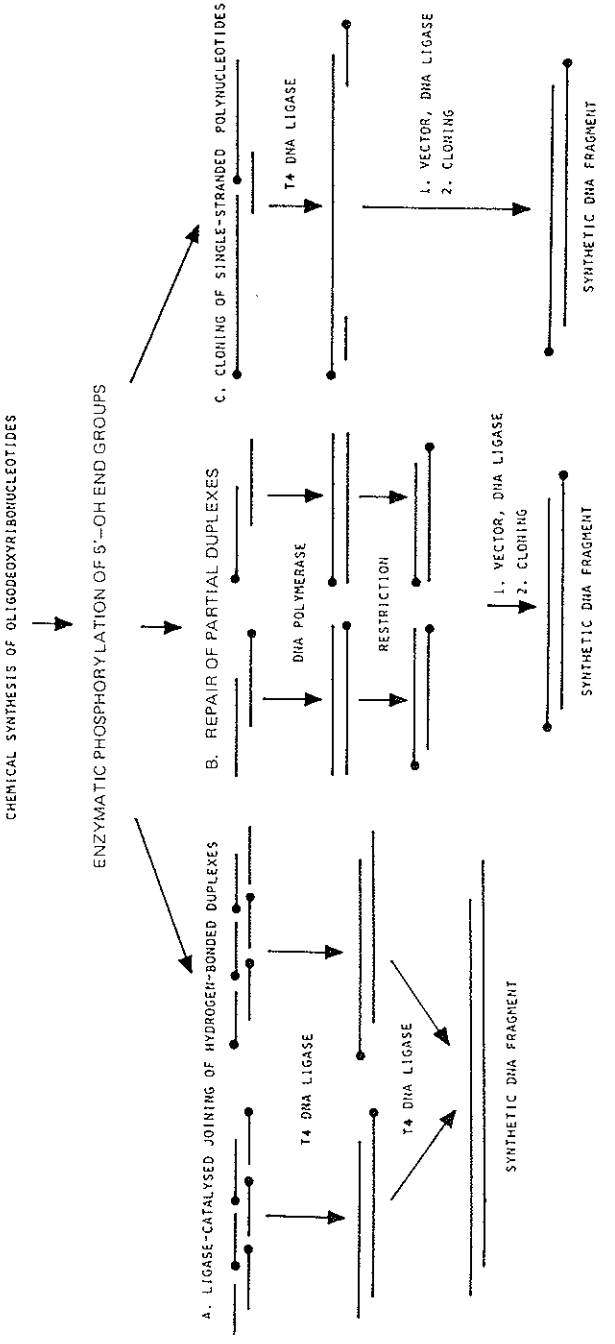


Figure 5. Three approaches to the synthesis of double-stranded polynucleotides. ●— represents 5'-phosphorylated oligonucleotides.

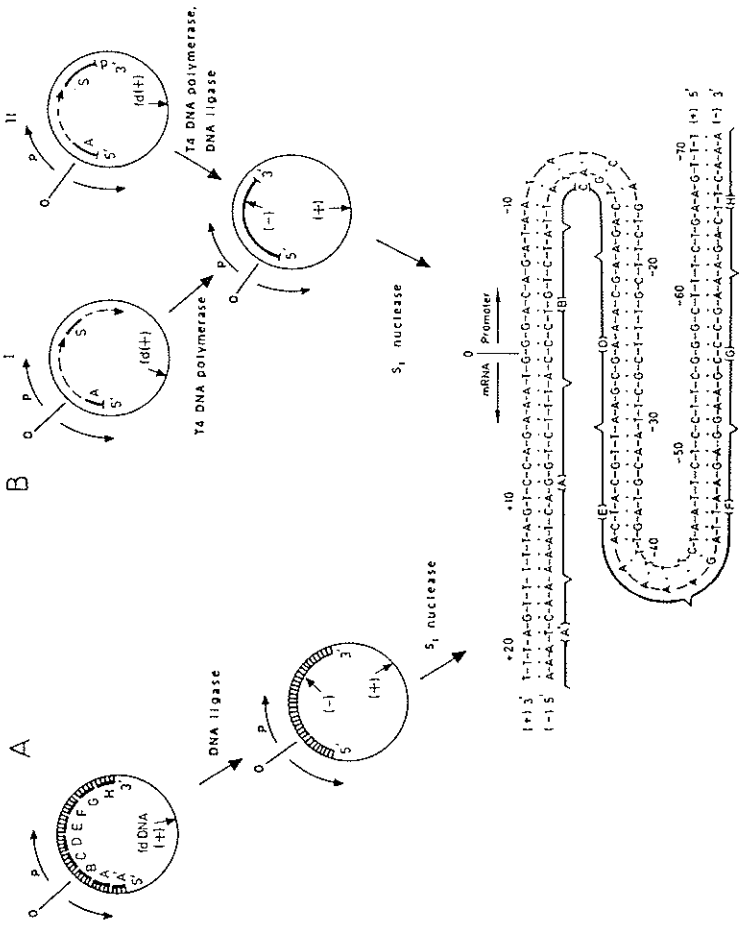


Figure 6. Approaches to synthesis of a promoter region of phage fd DNA.

cedure has been proposed involving repair synthesis by DNA polymerase I (EC 2.7.7.7) of a partial duplex formed by the 3′-terminal sequences of two long oligonucleotides, resulting in a completely double-stranded DNA segment. The latter was purified by cloning after formation of restriction sites at the ends (Itakura, 1982) (*Figure* 5(B)). Preparation of a 132 bp fragment of the gene for human leukocyte interferon α_2 is an example of the practical use of the approach (Rossi *et al.*, 1982).

In 1985 another novel procedure for constructing duplexes was described. This suggested the synthesis of only one of the chains of the target DNA fragment plus two oligonucleotides complementary to the 5′- and 3′-termini of this chain and forming restriction sites. The partial duplex, formed by the single-stranded synthetic DNA and these oligonucleotides, is cloned without repair *in vitro*. After isolation from the vector, the target DNA fragment can be obtained as a fully double-stranded polynucleotide (Amirkhanov *et al.*, 1985) (*Figure* 5(C)). The potency of the method was demonstrated by cloning a single-stranded polynucleotide 93 nucleotides in length, representing the leader sequence of the human fibroblast interferon gene.

All the above methods are usually quite reliable and effective in the synthesis of relatively short DNAs, on average 100–300 bp long. As described in some publications (Brousseau *et al.*, 1982; Ikehara *et al.*, 1984), it seems more feasible to assemble longer genes from preliminarily cloned separate modular units (or subfragments). This facilitates the assembly and purification procedures and minimizes the amount of synthetic work. For successful cloning the synthetic gene subfragments should carry appropriately positioned restriction sites unique for the gene and for the cloning vehicle. In general, sites present in the gene sequence are used. However, the number of useful sites can be restricted, thus limiting the possibilities of this approach to synthesis of double-stranded DNAs, necessitating the development of specific techniques for constructing longer genes. Attempts were made to improve the procedure for assembly of long double-stranded polynucleotides: for instance, the use of retrieval adapters carrying sites for the restriction endonucleases *Mbo* II, (Narang *et al.*, 1980) and *Hga* I (Korobko *et al.*, 1982). These endonucleases cleave DNA several nucleotides away from the recognition site. The gene fragment isolated from the vector molecule after cloning by means of such enzymes has at its ends unique protruding sequences promoting its conjunction with other target gene fragments cloned in the same way. However, this method has some disadvantages, such as the use of expensive restriction enzymes and the considerable amount of additional synthetic work involved. Apparently more promising is the cloning of subfragments in pBBV plasmid vectors carrying sites for endonuclease *Bbv* II, which also acts like the enzymes described above (Dobrynin *et al.*, 1984b). This technique also has some drawbacks as it requires special vectors and a restriction endonuclease which is hard to obtain.

Recently, a general approach for assembling double-stranded polynucleotides, which permits synthesis of DNAs of practically unlimited lengths and sequences, has been elaborated (Chakhmakhcheva *et al.*, 1985). The DNAs are constructed from preliminarily cloned modular units (subfrag-

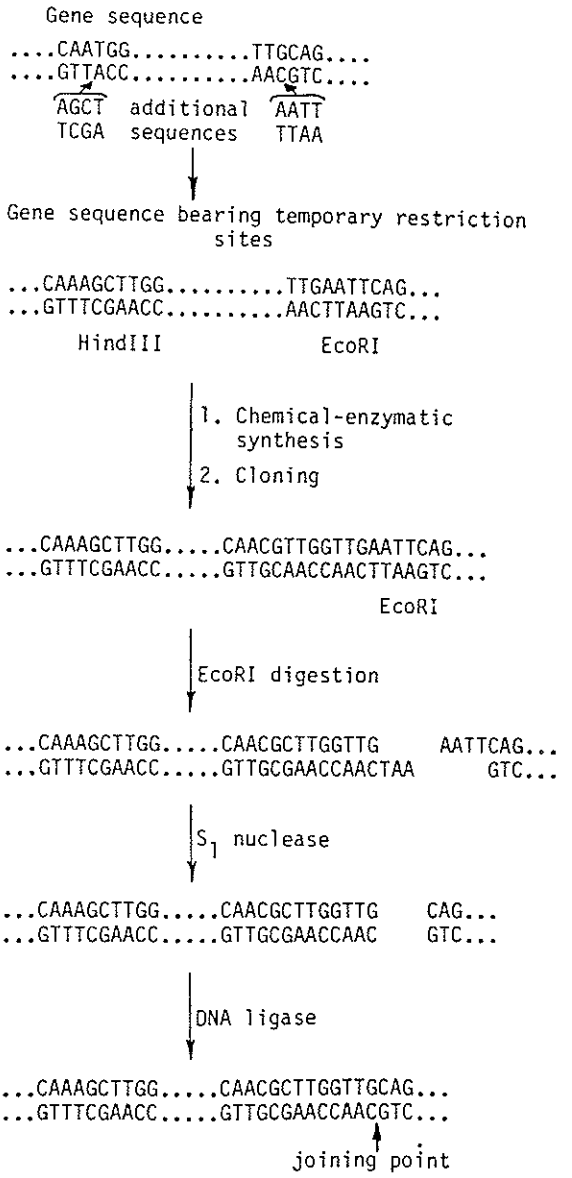


Figure 7. Scheme for introduction of temporary restriction sites into the DNA sequence to be synthesized and their removal during synthesis.

ments) using temporary restriction sites for cloning individual subfragments and assembling the target polynucleotide. The points for insertion of temporary restriction sites are chosen during the experimental planning stage and form the boundaries of the modules from which the gene will be finally assembled. *Figure 7* illustrates the insertion and removal of such restriction sites. Two proposed methods for assembling the DNA fragments by means

of temporary restriction sites, both utilizing vectors carrying polylinker sequences with a set of unique restriction sites, are shown in *Figure 8*. The efficacy of this approach has been proved by the synthesis of functionally active DNAs, in particular of a functionally important fragment of the bacteriorhodopsin gene (Chakhmakhcheva *et al.*, 1985), and of the promoter region P_L of bacteriophage λ. This method has also been used for the synthesis of the proinsulin, human preproinsulin (Ovchinnikov *et al.*, 1984a), and human interferon α_2 genes.

Chemical-enzymatic synthesis of artificial genetic structures

The intense development of molecular biology, especially genetic engineering, has strongly increased the importance of the chemical-enzymatic synthesis of extended nucleic acid fragments, in particular of genes for biologically active peptides and proteins. The synthetic approach, although time-consuming, has in some respects greater potential than that using cloned cDNAs or genome sequences isolated from natural sources. It allows the preparation of double-stranded polynucleotides of predetermined structure and the programming of synthetic DNAs, e.g. incorporation of conveniently placed sites for restriction endonucleases, or of transcription and translation regulatory signals. Codons favourable for enhancing the synthesis of corresponding mRNAs and proteins in the cell can be used for planning the sequences of synthetic genes. The synthetic approach simplifies directed mutagenesis and holds promise for obtaining analogues of a protein with alterations at functionally important positions.

In 1976 two small synthetic genes were cloned: the *lac* operator from *E. coli* (Heyneker *et al.*, 1976) and the gene for tyrosine suppressor tRNA (Khorana *et al.*, 1976). These studies first demonstrated that biologically active DNA fragments can be constructed by chemical-enzymatic synthesis with subsequent cloning. Both the genes, copies of the natural sequences, were active *in vivo*. In 1976 a synthetic gene for somatostatin was obtained (Heyneker *et al.*, 1976); the sequence was deduced from the primary structure of the peptide and from the genetic code. When the artificial gene was shown to be capable of expression *in vivo*, with the production of biologically active peptide hormone, unlimited perspectives for the design of synthetic genes became obvious in genetic engineering and biotechnology. This gave impetus to investigations where artificial double-stranded DNA fragments were employed in different ways. Genes investigated included those for biologically active peptides and hormones, such as somatostatin (Itakura *et al.*, 1977), insulin A and B chains (Goeddel *et al.*, 1979b), proinsulin (Brousseau *et al.*, 1982), interferons (Edge *et al.*, 1983; Tanaka *et al.*, 1983; Jay *et al.*, 1984), growth hormone (Ikehara *et al.*, 1984) and other genes (Rink *et al.*, 1984; Sproat and Gait, 1985; Tanaka *et al.*, 1982). Regulatory DNA fragments, in particular, promoter regions (Windass *et al.*, 1982; Munson *et al.*, 1984) were also investigated.

Studies on the directed synthesis of functionally active artificial DNA fragments were first performed in the USSR early in the 1970s. More recently

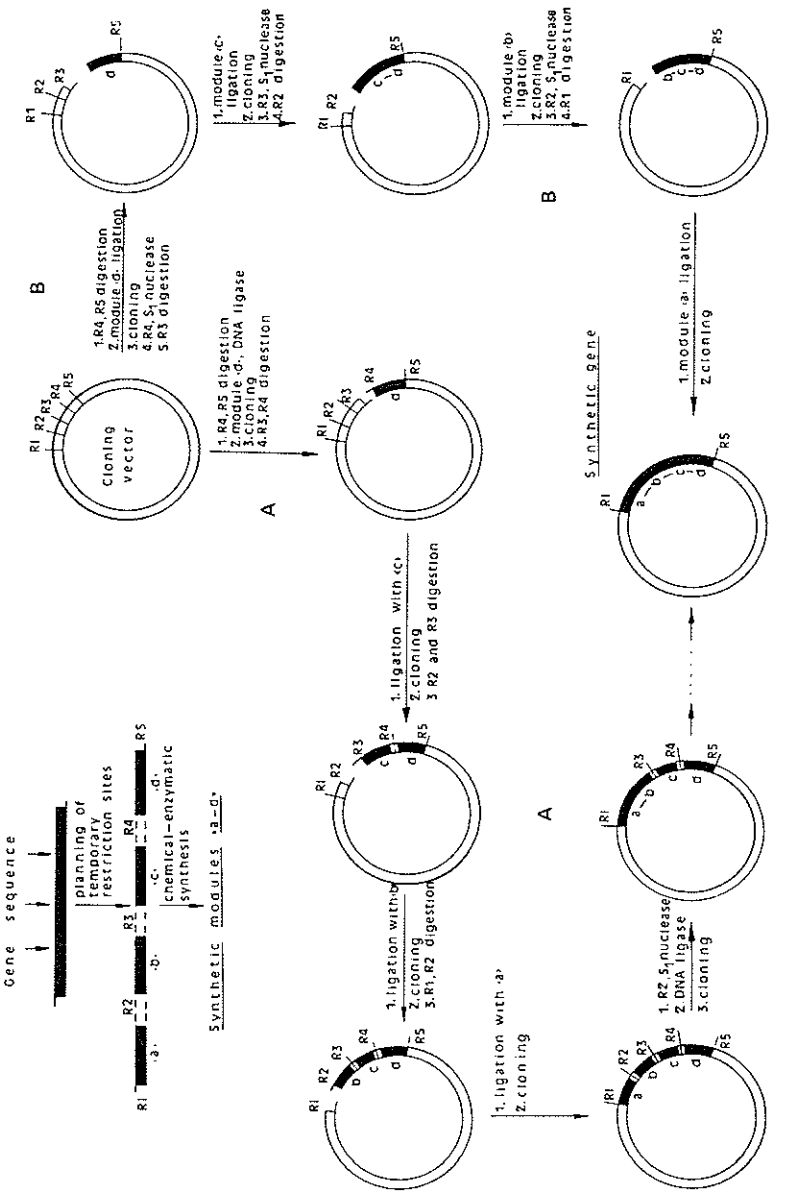


Figure 8. (Legend on page 93, opposite).

a series of genetic structures which can be divided into groups according to their functions have been designed. First, there are the genes encoding short peptides or RNA, e.g. leu-enkephalin (Efimov and Chakhmakhcheva, 1979), bradykinin (Dobrynin *et al.*, 1979), δ-sleep peptide (Dobrynin *et al.*, 1981), valine tRNA of yeast (Berlin *et al.*, 1983), angiotensin (Kumarev *et al.*, 1980), and the antigenic determinant of the surface antigen protein of hepatitis B virus (Smirnov *et al.*, 1983). Some of these sequences are shown in *Figure 9*. More recent syntheses include the calcitonin gene (Rubtsov *et al.*, 1984), fragments of the antigenic determinant of the foot-and-mouth disease virus (Korobko *et al.*, 1984), a fragment of the bacteriorhodopsin gene (Chakhmakhcheva *et al.*, 1985), as well as the large genes for human proinsulin (Ovchinnikov, Efimov and Chakhmakhcheva, 1983) and human interferon α_2 (*Figure 10*).

The second group of synthetic DNAs comprises regulatory regions, in particular, synthetic Shine–Dalgarno sequences (Efimov *et al.*, 1983a), terminators and synthetic operator (Korobko *et al.*, 1981a,b) and promoter regions (Ovchinnikov, Efimov and Chakhmakhcheva, 1979; Dobrynin *et al.*, 1980; Korobko *et al.*, 1980). In *Figure 11* sequences of a few promoters are presented: the ideal (consensus) promoter, promoter P25 of bacteriophage T4 DNA and their hybrids (Dobrynin *et al.*, 1980, 1984a). All the synthetic regulatory DNA fragments have been used successfully in the expression of synthetic and natural genes: for example, direct expression of the gene for human proinsulin was performed under the control of a synthetic bacteriophage fd promoter (Efimov *et al.*, 1984). *Figure 12* outlines the completely designed synthetic gene for human proinsulin containing a promoter sequence, the structural gene for the pro-hormone, and regulatory translation initiation and termination signals. The ideal promoter and its derivatives were used to express the genes for bradykinin, δ-sleep peptide and interferon, etc. (Korobko *et al.*, 1981c).

Figure 8. Strategy for construction of a synthetic gene from four subfragments with the use of temporary restriction sites. R1–R5: restriction endonucleases and corresponding restriction sites. (A) The first assembly route involves successive cloning of DNA subfragments in a suitable cloning vehicle, so that finally a complete gene, carrying all the inserted temporary restriction sites, can be assembled in the vector. To remove these sites, the plasmid DNA is also successively digested by corresponding restriction endonucleases and treated by S_1 nuclease. In this case, it is very convenient to assemble the gene from synthetic subfragments supplied with cohesive ends carrying half-sites of restriction endonucleases. (B) The second (more rapid) assembly route involves consecutive joining of modular units of genes with simultaneous removal of temporary restriction sites. Cloning the first subfragment is performed in the same way as for route A. After digestion by the corresponding restriction enzyme, the temporary site at the end of this plasmid-incorporated module is removed by S_1 nuclease action and then a cohesive terminus for insertion of the second module is generated at the other end of the molecule. The second synthetic subfragment, with one blunt and one cohesive end, is introduced into the plasmid vector prepared in this way. The blunt end is employed for joining to the preceding subfragment, which has been cloned already, while the cohesive terminus is joined to the second end of the plasmid vector. At the junction of the two modules a sequence is formed corresponding to the exact structure of the gene.

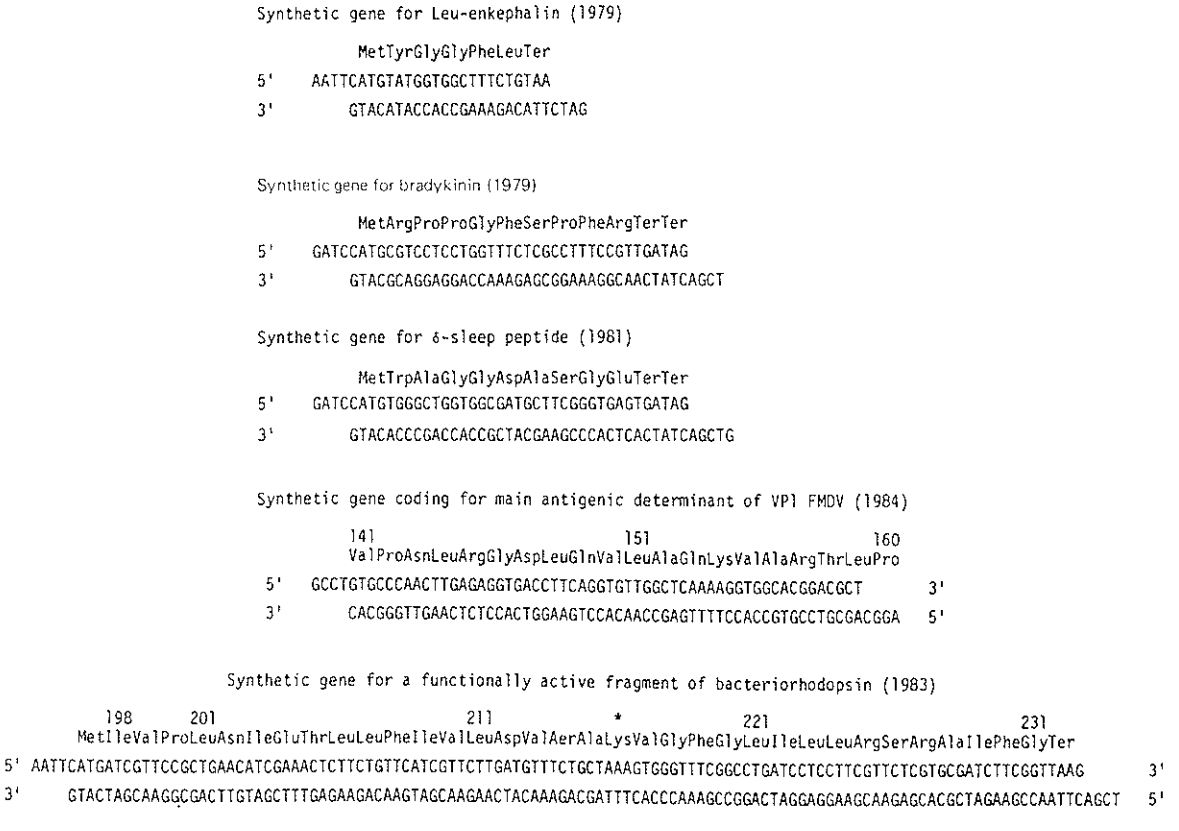


Figure 9. Sequences of synthetic genes for several biologically active peptides and their amino-acid sequences.

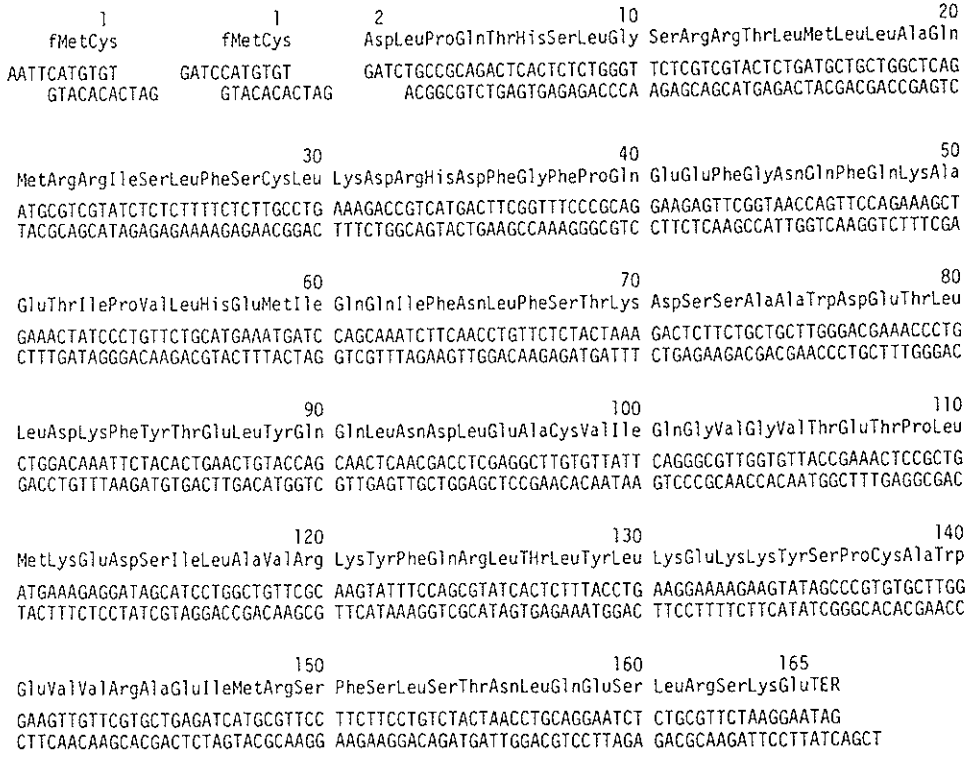


Figure 10. The sequence of a synthetic gene for human interferon α_2 and the amino-acid sequence of this protein.

Figure 11. Synthetic ideal promoter and its combinations with the promoter region P_{25} of phage T4.

NATURAL GENE EDITING

Although a gene of any size can, in principle, be made by modern chemical-enzymatic DNA synthesis methods, in some cases isolation of the corresponding natural gene and its adaptation for future work by the introduction of several synthetic DNA fragments seems more practical. The efficacy of this approach was first demonstrated with growth hormone (Goeddel *et al.*, 1979a). As a rule, genes produced by reverse transcription of mRNA or isolated from genomic sequences do not contain the whole desired sequence, or they have additional sequences at the ends, and introns that sometimes prevent their application in genetic engineering and biotechnology. Here, 'editing' with fragments of synthetic DNAs seems reasonable, especially with fragments which complete the gene from the first suitable restriction site to the 5′-end. If there is no such site in the natural gene sequence, it can be inserted by site-directed mutagenesis (*see below*), and after attachment of the synthetic duplex this site can be removed. This approach is now applied widely, for instance, in the expression of genes for some interferons (Ovchinnikov *et al.*, 1982b; Sverdlov *et al.*, 1984), for tumour necrosis factor (Shirai *et al.*, 1985), and for β-galactosidase (Korobko *et al.*, 1983).

Use has been made of synthetic DNA fragments for constructing vectors suitable for the expression of small peptides as fusion proteins with β-galactosidase (Efimov *et al.*, 1984; Guo *et al.*, 1984). An example is shown in *Figure 13*. Plasmid pG1005 carrying the larger portion of the gene for *E. coli* β-galactosidase, with its promoter and operator, was treated with endonuclease *Eco* RV, yielding a β-galactosidase gene fragment coding for 380 amino-acid residues from the *N*-terminus, having blunt ends unsuitable

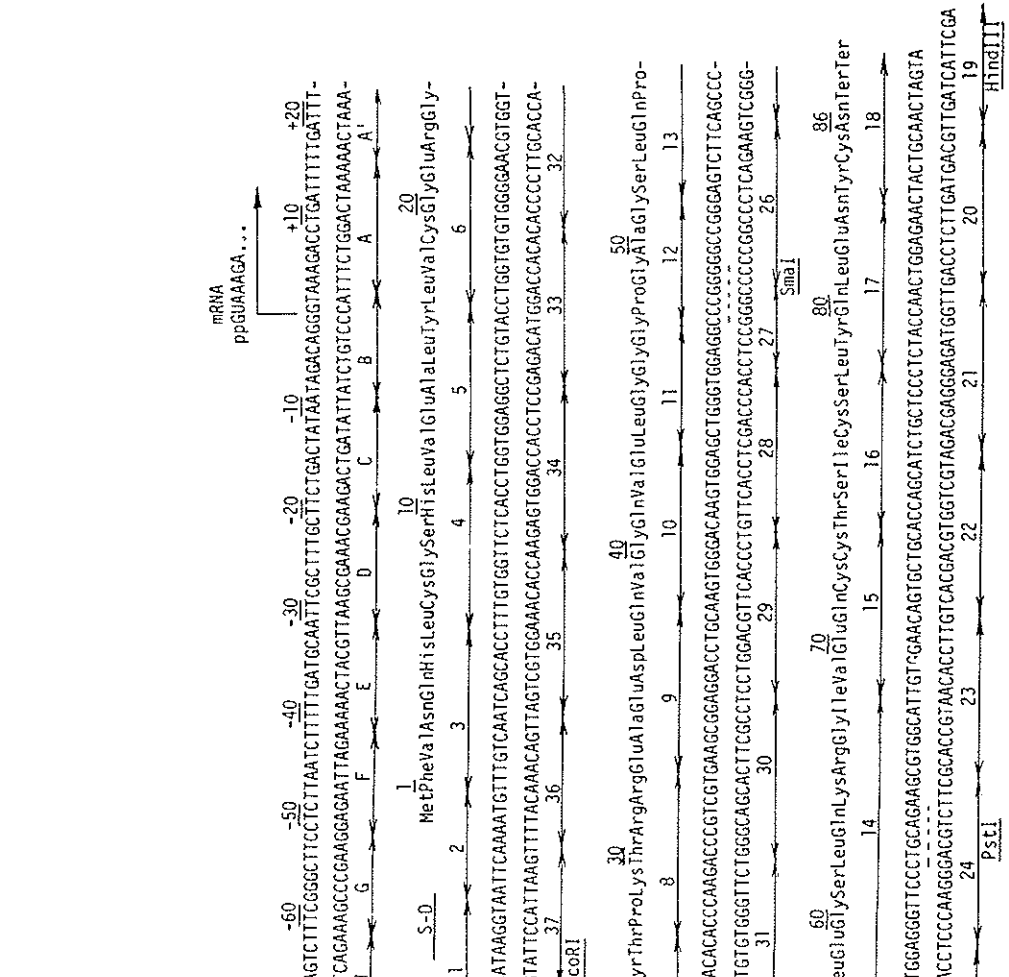


Figure 12. The structure of the fully synthetic functional gene for human proinsulin and its amino-acid sequence.

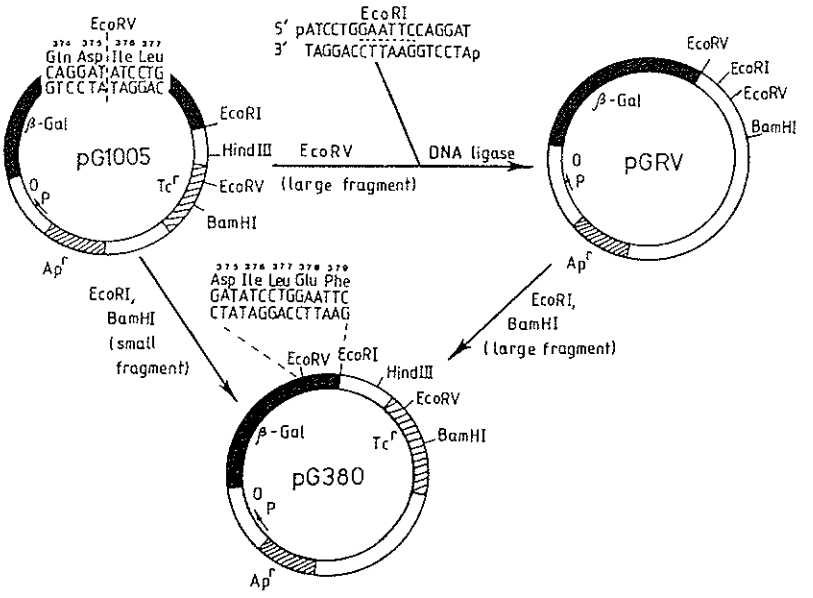


Figure 13. Construction of a vector for expression of genes for short peptides in *Escherichia coli*.

for attachment to the genes to be expressed. The addition of a small synthetic linker containing the *Eco* RI site yielded a vector (pGRV) suitable for expressing short peptides in *E. coli*, with the hybrid protein under the control of the regulated promoter-operator system. Other expression vectors have been produced from fragments of the β-galactosidase gene of various lengths (Efimov *et al.*, 1984). Thus synthetic DNA fragments may also be used in genetic engineering to design novel vector molecules.

CONSTRUCTION OF NOVEL VECTORS

A series of plasmid and phage vectors suitable for cloning, sequencing and expressing genes has been constructed by means of synthetic oligo- and polynucleotides. At present there is a family of vectors based on bacteriophage M13 DNA, which is constantly being increased in number (Messing, 1983). These molecules contain synthetic sequences carrying a set of endonuclease restriction sites. For cloning, sequencing and expressing heterologous genes in *E. coli*, several plasmid vectors of the pUC family have been produced which contained polylinker synthetic DNA regions (Vieira and Messing, 1982). In the USSR new vectors have also been created using synthetic sequences, e.g. vectors of the pBBV type mentioned above (Dobrynin *et al.*, 1984b), and the pHS (Efimov *et al.*, 1985b) and pPLE vectors (Chakhmakhcheva *et al.*, 1985) (*Figure 14*). To obtain the pHS and pPLE vectors, plasmid pBRSI (Ovchinnikov, Efimov and Chakhmakhcheva,

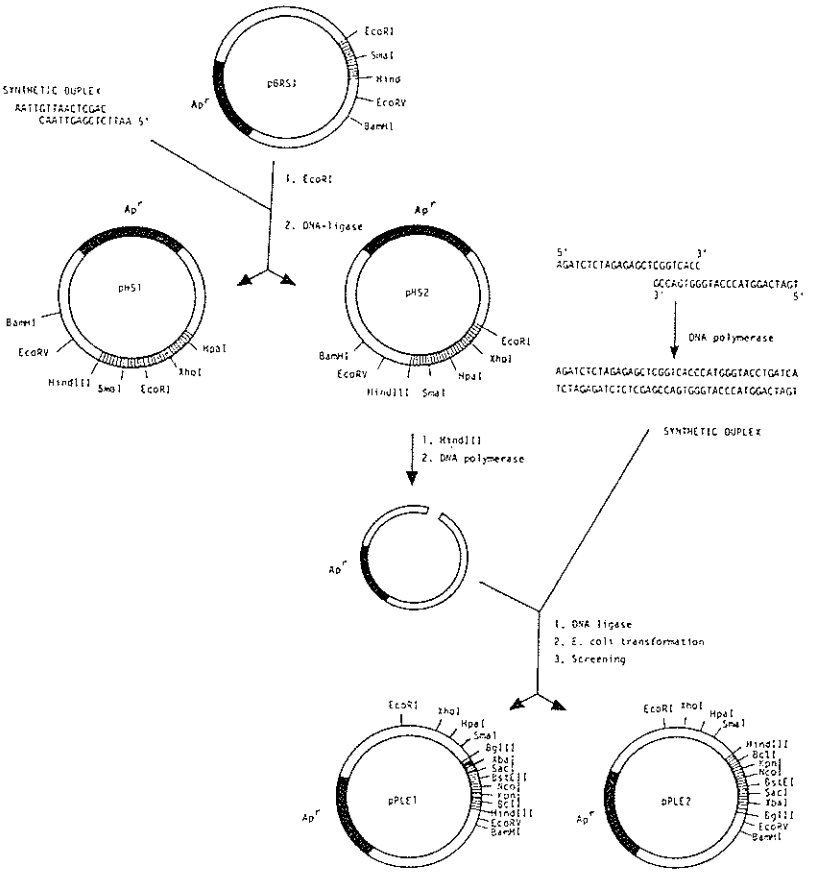


Figure 14. Construction of novel vectors for cloning, synthesis and site-directed mutagenesis of DNA fragments.

1983) was made suitable for cloning, oligonucleotide-directed mutagenesis and construction of synthetic genes, by the insertion of synthetic duplexes of 15 and 40 bp in length. These molecules contain several unique restriction sites within the relatively short DNA region. The application of synthetic oligonucleotides resulted in the original phage vectors M13mp18amIV and M13mp19amIV, designed especially for site-directed mutagenesis of DNA fragments (*see below*) (Carter, Bedouelle and Winter, 1985).

Application of oligodeoxyribonucleotides for isolating and cloning natural DNA fragments

The ability of synthetic oligonucleotides to hybridize with complementary sequences on DNA and RNA templates allows the isolation and identification of naturally occurring genes. Oligonucleotides can be used in a variety of ways for investigations of this kind.

PRIMERS

Nowadays, oligodeoxyribonucleotides 15-17 nucleotides in length are widely employed to determine the primary structures of DNA and RNA by the 'dideoxy' chain termination method (Sanger *et al.*, 1982; Brenner and Shaw, 1985) with the use of pure RNA or cloned DNA as templates. The sequence is read from the positions of radioactive bands on a polyacrylamide gel, which are the terminated products of primer elongation.

Oligonucleotides are also used for selective priming of mRNA. In order to do this, it is necessary to know the sequence of the small fragment of the desired nucleic acid (RNA), or at least the sequence of 5–6 amino-acid residues of the encoded protein. The first use of specific priming for the molecular cloning of a gene was to determine the structure of the rat proinsulin gene (Villa-Komaroff *et al.*, 1978); this approach is now extensively exploited. Numerous examples of its use include the isolation and cloning of human interferon genes carried out in the USSR (Ovchinnikov *et al.*, 1982a, 1983, 1984b; Gren *et al.*, 1983). In these investigations synthetic 16-mers were widely used, not only as specific primers but also as hybridization probes for identifying cloned DNA (*see below*). Similar methodology was of value in isolating the genes for bacterial and bovine rhodopsins as well as other proteins (Zozulya, Zaytseva and Sverdlov, 1984).

When no information on the structure of the DNA or RNA concerned is available, but the structure of at least a small fragment of the encoded protein is known, the so-called mixed probe-primers can be used. The structures of the latter are derived from the genetic code, taking into account all possible variants of the nucleotide sequence (Houghton *et al.*, 1980).

HYBRIDIZATION PROBES

Synthetic nucleotides may also be used as hybridization probes for the isolation and identification of genes, in particular for eukaryotic DNA. The complex structure of eukaryotic genomes necessitates the screening of a large number of clones in order to localize the specific DNA sequence desired. To facilitate the procedure, selection of recombinant phage or plasmids is performed by hybridization with homologous labelled nucleic acid fragments—mRNA preparations or rather large DNA fragments isolated from natural sources. However, natural fragments of nucleic acids are seldom, if ever, accessible in a pure state or in sufficient amounts. Here the indispensable molecular probes are synthetic oligonucleotides, with structures corresponding to the sequence of one of the regions of the desired DNA fragment (Suggs *et al.*, 1981).

The hybridization method depends on the ability of polynucleotides to recognize complementary DNA sequences and to form double-stranded complexes with them, and on the reduced stability of oligonucleotide complexes with partially complementary sequences. Two modifications of this method have been described. One involves preliminary isolation of DNA from numerous clones or phage plaques and their subsequent hybridization

with a labelled oligonucleotide probe. This is laborious, because hundreds or even thousands of DNAs must be isolated. A better procedure is hybridization with the probe *in situ* on the surface of nitrocellulose or paper filters on to which the bacterial clones carrying recombinant DNAs have been replica-plated. Thus several thousands of colonies can be screened simultaneously, and those which contain DNA fragments homologous to the sequence of the molecular probe can be rapidly identified.

After immobilization on filters, libraries created by reverse transcription of mRNA, or genomic libraries prepared by any 'shot-gun' technique can be screened with synthetic, labelled probes. Their optimal lengths depend on numerous parameters, in particular on the size of the gene bank to be screened (Efimov and Chakhmakhcheva, 1982). Relatively short probes (14 to 17-mers) have advantages compared with longer oligonucleotides as even a difference of 2 bp between the probe and DNA strongly influences the stability of the complex. With regard to the 'mixed' probes, the efficacy of screening depends heavily on hybridization conditions and on the number of individual oligonucleotides in the mixture. Usually, probes with degeneracy from 8 to 32 are used. Application of more degenerate mixtures can also be a success, although rarely. For example, a mixed probe containing 256 20-nucleotide oligomers has been used effectively for selection of clones containing serum amyloid P component cDNA (Mantzourams *et al.*, 1985). A mixture of 64 17-residue oligonucleotides has also been used to isolate and establish the primary structure of the Na^+,K^+-ATPase gene from pig kidney (Ovchinnikov *et al.*, 1985; Petrukhin *et al.*, 1985) (*Figure 15*). It

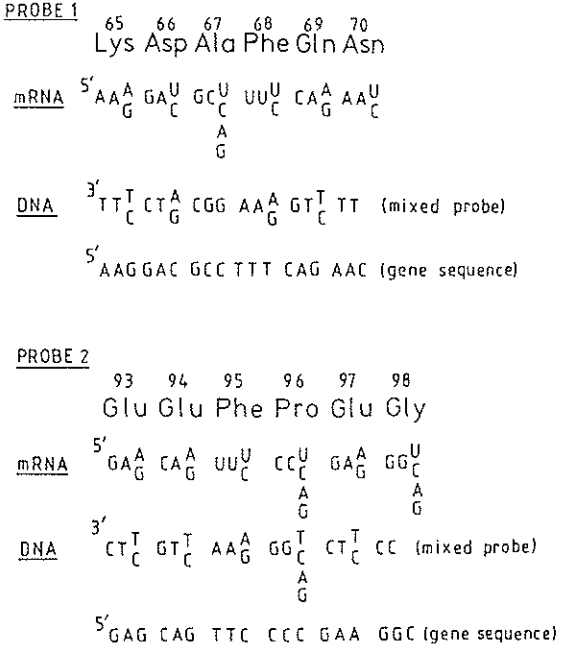


Figure 15. Mixed sequence probes designed for isolation of the gene for Na^+, K^+-ATPase from pig kidney.

should be noted that long synthetic fragments of DNA, with a unique sequence deduced from the peptide sequence and a statistical analysis of the usage of certain codons in the organism whose DNA library is to be screened, are an alternative for screening genomic libraries (Jaye *et al.*, 1983). Such long probes can be obtained either by direct chemical synthesis or by chemical-enzymatic approaches as described above. For instance, an 86-mer probe has been effectively exploited for isolation of the bovine pancreatic trypsin inhibitor gene from a genomic library (Anderson and Kingston, 1983).

LINKERS

Synthetic oligonucleotides can also be used as 'linkers' for cloning artificial and natural DNAs. The restriction sites can easily be attached to blunt-ended DNA sequences by means of a symmetrical linker which is a self-complementary oligonucleotide. The latter usually contains one or more restriction sites of this kind. Sometimes the linkers have two oligonucleotides of different lengths composing a short duplex with one blunt end and one sticky end (having the sequence of one of the restriction sites). To replace restriction sites at the ends of the DNA fragment of interest, linkers with two sticky ends are usually employed. Such synthetic DNA fragments are useful for the transition of vector molecules from one type to another in gene cloning and expression.

Site-directed mutagenesis

Synthetic oligo- and polynucleotides are widely used to insert mutations directly into DNA sequences. One technique utilizes insertion of a synthetic duplex containing changes in both strands, into a gene where the corresponding wild-type sequence is deleted, e.g. by treatment with restriction endonucleases. Another technique concerns the synthesis of a whole gene containing all the necessary mutations. Recently, Grundstrom *et al.* (1985) have tested this method by synthesizing a DNA fragment representing the simian virus 40 (SV40) enhancer. The functional role of certain sites of this DNA fragment was elucidated by careful mutational analysis, facilitated by the development of a special 'shot-gun' procedure for ligation and cloning of the mutant sequences. Such mutational analyses were also undertaken earlier in the study of the *lac* operator by Goeddel *et al.* (1978): a series of mutant *lac* operators was produced by chemical-enzymatic synthesis and used to study the interaction between the repressor and DNA in this system. Similarly, the functioning of promoter regions has been investigated to optimize gene expression. However, more frequently a mutagenesis technique is employed which allows insertion of site-directed mutations in previously synthesized polynucleotides or naturally occurring DNAs.

Generally, mutational changes in natural DNAs of known base sequence can be effected in a number of ways (Harris, 1982), but the most precise and suitable technique for insertion of specific mutations in cloned DNA fragments is through the use of synthetic oligodeoxyribonucleotides as site-

specific mutagens. Oligonucleotide-directed mutagenesis uses a synthetic oligonucleotide carrying the desired mutation (point mutation, deletion, or insertion) as a primer to direct DNA synthesis on a single-stranded circular DNA template. The oligonucleotide is incorporated into the second DNA strand. After ligation, transformation of this closed circular heteroduplex DNA into *E. coli* cells, followed by *in vivo* DNA replication, resolves this heteroduplex into the mutant and original, wild-type genes. The mutant can be easily identified against a background of non-mutant DNAs by hybridization with the same oligonucleotide (Smith, 1982).

Primer mutagens are usually 12–20 nucleotides in length but sometimes mutagenesis, including deletion of DNA regions, requires longer oligonucleotides. Deletions of short sequences with the use of a 20-mer have been described by Chan and Smith (1984). An 18-mer has also been employed for the deletion of a 1143 bp DNA fragment (Osinga *et al.*, 1983).

To carry out oligonucleotide-directed mutagenesis, two systems are generally utilized: single-stranded DNA phages (such as bacteriophage M13 and its derivatives) (Zoller and Smith, 1983) and partially or completely single-stranded circular plasmid DNAs. Initially, the latter were produced from supercoiled double-stranded plasmid DNA by introducing nick(s) into one of the plasmid chains using restriction endonucleases in the presence of ethidium bromide, followed by digestion of the nicked strand with endonuclease (Dalbadie-McFarland *et al.*, 1982). The yields of mutants were low, averaging 0·01–1·0%. Single-stranded DNA phages were more effective, but this approach also had disadvantages which decreased the yield of mutants. Moreover, in both cases, the use of DNA polymerase for primer elongation on the extensive, completely single-stranded circular template led to 'false' mutations. The yield of the desired mutation decreased owing to the presence of many DNA molecules with an incompletely repaired second strand in the reaction mixture, as DNA polymerase had difficulty in passing over template regions with secondary structure, in particular, the origin of replication.

Recently, special highly efficient approaches to mutagenesis using phage DNAs have been developed: the application of double-primer systems (Norris *et al.*, 1983), the 'gapped-duplex' DNA approach (Kramer *et al.*, 1984), and recloning of mutant fragments after repairing and cutting out the double-stranded mutated fragment with restriction endonucleases (Sims *et al.*, 1985). Thus, a method has been described for the construction of mutations in modified M13mp18 and M13mp19 vectors (Carter, Bedouelle and Winter, 1985). The DNA was first cloned into a novel M13 vector with a genetic marker or amber mutation in an essential phage gene. In this 'coupled-priming' technique one primer was used to construct the silent mutation of interest, and the second to eliminate the selectable marker on the (−) strand of M13 DNA. After primer extension and ligation, the heteroduplex DNA was transfected into a repair-deficient strain of *E. coli* and selected against the (+) strand marker. Over 50 mutations were produced by this method with a yield of up to 70%.

Methods for the oligonucleotide-directed mutagenesis of plasmid DNAs have also been improved, e.g. the direct usage of double-stranded molecules

(Bruce Wallace, 1985). These mutagenesis techniques appeared to hold promise for studying the influence of structures, e.g. the (−10) and (−35) regions of the *lpp E. coli* promoter on gene expression (Inouye and Inouye, 1985). Promoter efficacy was shown to be increased by substitution of the above-mentioned sites by consensus sequences.

Recently a method for the insertion of mutations into cloned DNAs has been developed by Efimov *et al.* (1985a,b) (*Figure 16*). Its main points are as follows:

1. Specially constructed plasmid vectors for gene cloning and mutagenesis are used;
2. The super-coiled plasmid is digested with a blunt-ended restriction endonuclease;
3. The linear double-stranded DNA obtained is digested with *E. coli* exonuclease III, which under controlled conditions produces a duplex with protruding single-stranded 5′-ends which must include the site of the proposed mutation (degradation of DNA outside the mutagenesis region is kept to a minimum);
4. The synthetic oligonucleotide carrying the desired mutation is annealed with plasmid DNA prepared as described above, the single-stranded regions are filled in with DNA polymerase and the resultant heteroduplex is circularized with DNA ligase.

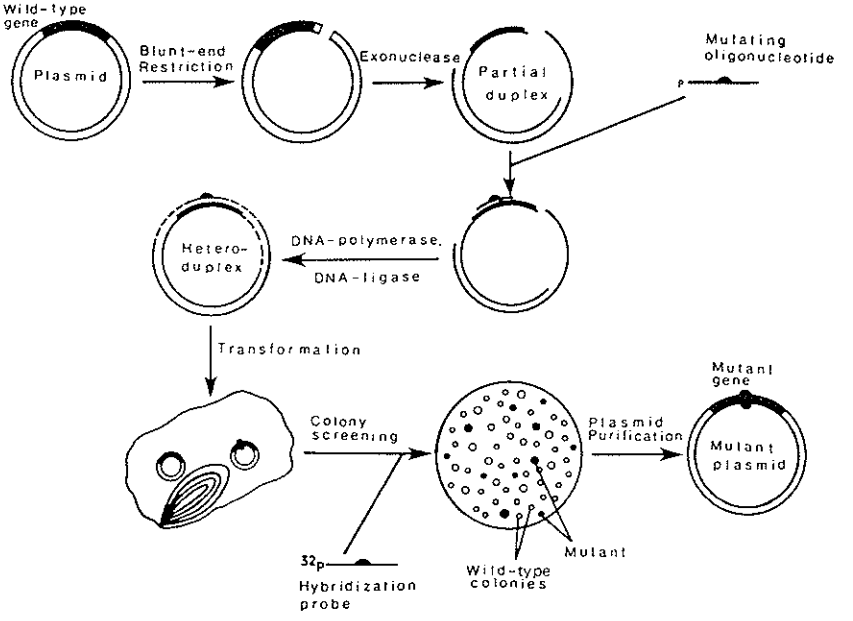


Figure 16. Overall scheme for oligonucleotide-directed mutagenesis of double-stranded linear plasmid DNA.

An increase in the yield of mutant DNA of up to 10–20% of the total amount of non-mutant plasmids was found. This technique was used to introduce mutations into the human proinsulin gene, the gene X promoter of bacteriophage fd and other cloned synthetic DNAs. This procedure can be extended to introduce an insertion into comparatively short genes cloned in any plasmid vector if the gene to be mutated contains a unique site for a restriction endonuclease which generates blunt ends. The method can be also applied to mutagenesis of double-stranded RF DNA of phages (M13mp8, M13mp9, etc.) with polylinker sequences suitable for cloning and providing unique sites for restriction enzymes producing blunt ends.

PROTEIN ENGINEERING

Recent work has shown the first successes in the application of site-directed mutagenesis for studying structure–function relationships in proteins (Ulmer, 1983; Estell, Graycar and Wells, 1985; Estelle *et al.*, 1985). Gene mutations permit virtually any modification in the amino-acid sequences of the corresponding proteins, which might result in modified molecules with increased or novel biological activity. Together with data on the primary and higher structures of proteins, directed mutagenesis also shows which amino-acid residues are involved in active sites, in assembly of subunits, etc., i.e. it allows elucidation of the mechanism of protein action.

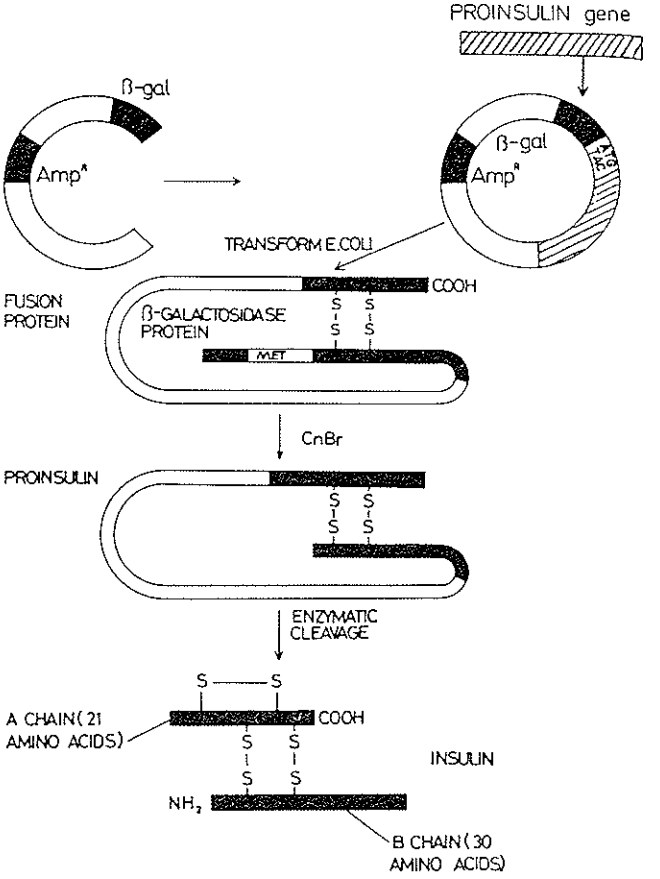
For making small changes in the protein structure (insertions, deletions or substitutions) it seems reasonable to employ one of the above-mentioned procedures for oligonucleotide-directed mutagenesis of genes. The procedure has the disadvantage that only one modification at a time can be produced, but it requires the least amount of chemically synthesized DNA and will therefore be the method of choice for most initial attempts at protein engineering. Indeed, this approach has been used to modify the active site of tyrosyl-tRNA synthetase (Fersht *et al.*, 1984). As a result of oligonucleotide-directed mutagenesis of the gene for this enzyme, which was carried out using phage vectors, followed by expression of mutant DNAs in *E. coli*, protein molecules with altered activity, relative to the natural protein, for the substrate, adenosine triphosphate, were produced. These included mutant enzymes with increased activity (Wilkinson *et al.*, 1984). These studies demonstrate the success of directed mutagenesis.

As mentioned above, an alternative approach for inserting mutations into a protein is the chemical-enzymatic synthesis of an altered gene. The sequence of the artificial gene can be constructed at will, and the principles of synthesis simplify the subsequent modifications. As the gene is created from a number of oligonucleotides, one can introduce changes at several sites simultaneously. This approach is preferable when essential changes to the protein sequence are obvious. Mutant proteins obtained thus include interferon (Edge and Camble, 1984) and proinsulin.

The mini-C analogue of human proinsulin with a 35 bp fragment joining the insulin polypeptide chains A and B substituted by a sequence of six amino-acid residues was obtained in this way and expressed in *E. coli* (Wetzel

et al., 1981). The product of gene expression was used to study the folding of the proinsulin molecule and its conversion into insulin.

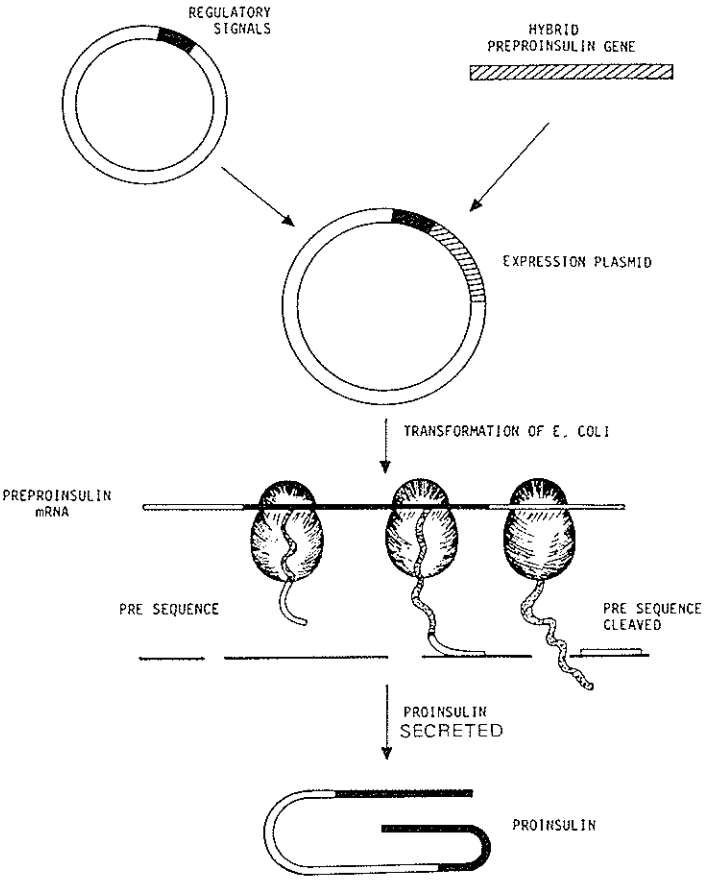
A combination of both approaches is also useful for obtaining mutant proteins. Oligonucleotide-directed mutagenesis and chemical-enzymatic synthesis have been utilized to insert specific amino-acid changes in bacteriorhodopsin (Lo *et al.*, 1984). A new unique restriction site (*Xho* I) was introduced into the gene for this protein by oligonucleotide-directed mutagenesis and a 62 bp restriction fragment was substituted by synthetic duplexes carrying various mutations. As a result six mutant bacteriorhodopsins were produced for a study of the mechanisms of bacteriorhodopsin function.



(a)

Figure 17. Schemes for obtaining human proinsulin in bacterial cells. (a) Expression of proinsulin as a fused protein with a fragment of *Escherichia coli* β-galactosidase. Proinsulin is released from the chimaeric protein translation product by reaction with cyanogen bromide. After purification and folding, proinsulin is enzymatically converted to human insulin. (b) Secretion of proinsulin by the engineered bacteria. The proinsulin gene is joined to the α-amylase gene signal sequence resulting in the expression of an α-amylase–proinsulin fusion protein. The latter is secreted from bacteria, because of the presence of the signal peptide on the *N*-terminal portion of the hybrid.

Protein engineering also deals with the production of peptide hormones by recombinant DNA technology (Wongenmayer, 1983), in particular with expression of genes for short peptides as fused proteins. Here the problem of rapid degradation by endogenous proteases of eukaryotic polypeptides expressed in bacteria has been overcome, resulting in biotechnological synthesis of these compounds. As leader sequences the fragments of some bacterial proteins are usually used: e.g. *E. coli* β-galactosidase, tryptophan synthetase (Williams, 1982) or *B. subtilis* α-amylase. *Figure 17* outlines the production of human proinsulin as a fused protein with a β-galactosidase fragment (*Figure 17a*) or with the signal peptide of α-amylase (*Figure 17b*). In the first case the fused protein forms insoluble aggregates in the cytoplasm which prevent degradation of the desired pro-hormone by proteases. In the second case the expressed fusion protein is subjected to processing and the proinsulin formed either accumulates in the periplasmic space (in the case of *E. coli* strains) or is secreted extracellularly (in the case of *B. subtilis* strains) (Efimov, Chakhmakhcheva and Ovchinnikov, 1984).



(b)

To facilitate isolation of the genetically engineered proteins, insertion of polyarginine fragments in the protein sequence has been suggested. This markedly changes the properties of the expressed protein and facilitates its separation from other bacterial proteins. After purification of the chimaeric protein, these additional sequences can be removed by treatment with carboxypeptidase B (Smith *et al.*, 1984).

Conclusion

Synthetic oligo- and polynucleotides of well-defined sequences have a number of important applications in molecular biology and recombinant DNA research. They have a key role in solving some of the problems of nucleic acid structure. They are widely used in genetic engineering as probes for the isolation of cDNA and genomic DNA clones, as primers in RNA and DNA sequence determination and as site-specific mutagens. Moreover, it is possible readily to produce and analyse direct structural modifications of proteins and nucleic acids with the help of synthetic DNA fragments, and this will be crucial in solving the long-standing problems of structure–function relationships in biopolymers. This information can then be applied to create novel enzymes, hormones and other useful proteins with improved properties for commercial applications. Thus, protein engineering paves the way for the microbiological production of peptides and proteins of great practical value.

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